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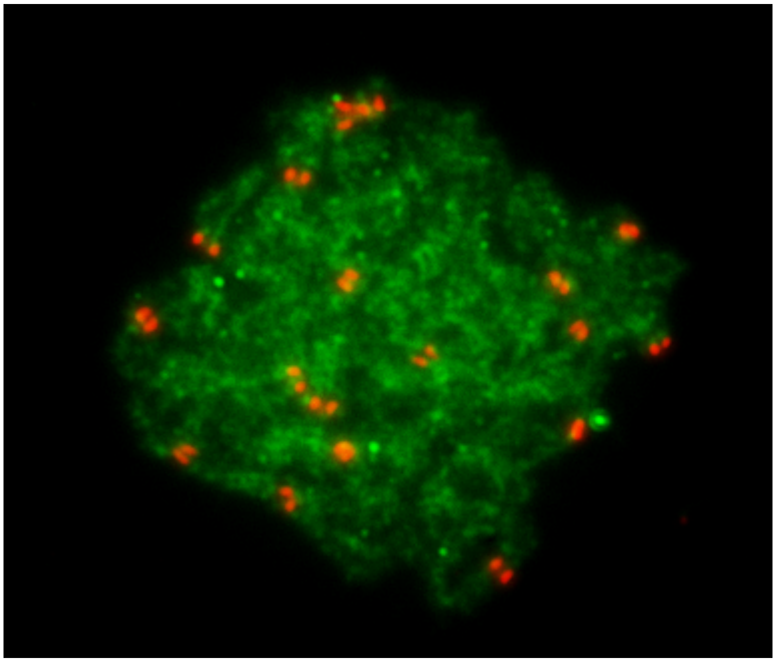
Crosstalk between kinetochore assembly and cohesion at centromeres



Samantha Jane Williams

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Contents

The centromere is the most critical chromosome region to ensure correct chromosome segregation. It is the position upon which the kinetochore machinery assembles and consequently microtubules can attach to separate sister chromatids in mitosis. It is also the region in which cohesion between sister chromatids, mediated by cohesin, is maintained until they are ready to separate at the end of mitosis. The centromere is epigenetically defined by the presence of a histone H3 variant known at centromere protein A (CENP-A). At least sixteen other different centromere proteins, CENPs, assemble upon CENP-A chromatin and are present throughout the cell cycle. These proteins are collectively known as the constitutive centromere associated network, the CCAN. The CENPs are divided in to various subcomplexes. Among them, CENP-C, CENP-T-W-S-X, CENP-L-N and CENP-H-I-K-M complexes have all been implicated in either centromere propagation or kinetochore assembly functions. The CCAN complexes have a complicated network of interactions and studies of the hierarchy of assembly in a variety of model systems have given contradictory results. In this Thesis we have used the *Xenopus laevis* egg cell-free system to investigate the hierarchy of '*de novo'* CCAN assembly as well as its role in the regulation of centromeric cohesion. Using biochemical methods and fluorescent microscopy we found that CENP-C and CENP-T drive two parallel pathways of kinetochore assembly. CENP-N and CENP-K mediate the crosstalk between these pathways, stabilising interactions in a cell cycle regulated manner. Both CENP-C and CENP-T are able to recruit Shugoshin 1 (Sgo1), the protein that protects cohesin from release specifically at the centromere during mitotic prophase. This is possible because either protein can independently recruit the entire KNL1/MIS12/NDC80 (KMN) kinetochore protein network, and Knl1 recruits Bub1. This kinase generates the phosphoH2A signal recognized by Sgo1. We found that forced targeting of Bub1 to centromeres in the absence of CENPs other than CENP-A restores Sgo1 recruitment. Importantly, we observed clear centromere cohesion defects in the absence of CENP-C, but not CENP-T, even though a similar amount of Sgo1 is recruited in both conditions. The reason for this defect is currently under investigation. While it does not seem to be the consequence of impaired cohesin deposition at centromeres, it could be related with the recruitment of condensin II. Alternatively, we also observed decreased amount of CENP-A in chromosomes lacking CENP-C. We speculate that the altered composition of centromeric chromatin could affect its architecture and lead to loosened cohesion.

El centrómero es un elemento cromosómico esencial para asegurar una segregación cromosómica correcta. Este locus dicta el ensamblaje del cinetocoro, una estructura multiproteica que media la interacción entre los cromosomas y los microtúbulos del huso para orquestar así la segregación cromosómica. Es también la región en donde las cohesinas mantienen el apareamiento de las cromátidas hermanas (o cohesión) en los cromosomas ya condensados. El centrómero está definidos epigenéticamente por la presencia de una variante de la histona H3 que se denomina CENP-A (de *Centromere Protein A*). Se ha descrito un grupo de dieciséis proteínas centroméricas que interaccionan directa o indirectamente con CENP-A, agrupadas en al menos 6 complejos distintos, que reciben colectivamente el nombre de CCAN (por *Constitutive Centromere Associated Network*). La jerarquía de interacciones físicas y funcionales entre los distintos componentes del CCAN que determinan el correcto ensamblaje y funcionamiento del cinetocoro está aún por determinar. En esta tesis hemos empleado un sistema *in vitro* basado en extractos obtenidos de huevos de *Xenopus laevis* en los que es posible ensamblar cromosomas con cinetocoros funcionales para entender dicha jerarquía, así como la contribución del CCAN a la regulación de la cohesión centromérica. Mediante técnicas bioquímicas y microscopía de fluorescencia, hemos explorado la interacciones entre cuatro componentes del CCAN, CENP-C, CENP-T, CENP-N y CENP-K, en cuanto a su dinámica de asociación a cromatina y su contribución a la formación *de novo"* del cinetocoro. Así, hemos encontrado que existen dos vías paralelas de ensamblaje del cinetocoro, una dirigida por CENP-C, y otra por CENP-T, conectadas y estabilizadas por CENP-N y CENP-K de forma distinta en interfase y en mitosis. Estas dos vías son también capaces de promover de forma independiente el reclutamiento de Sgo1, la proteína encargada de proteger a las cohesinas centroméricas de la disociación masiva que tiene lugar al comienzo de la profase. Este reclutamiento viene mediado por la fosforilación de las histona H2A por Bub1, quien, a su vez, se localiza a en la región centromérica a través de su interacción con el complejo Knl1 del cinetocoro externo. Hemos determinado que el cinetocoro sólo proporciona la plataforma donde aterriza Bub1, pues en su ausencia es posible reclutar Sgo1 al forzar la unión de Bub1 al centrómero. Una observación interesante es que la ausencia de CENP-C provoca defectos de cohesión mucho más severos que la ausencia de CENP-T, aún cuando la cantidad de Sgo1 presente en el centrómero es muy similar en ambas condiciones. No hemos encontrado evidencias de que CENP-C sea importante para la deposición de cohesina en la región centromérica o para que la cohesina se convierta en cohesiva. Sin embargo, hemos observado que CENP-C estabiliza a los nucleosomas CENP-A y en su ausencia la composición de la cromatina centromérica, y posiblemente su estructura, están alterados. Finalmente, hemos determinado que CENP-C, pero no CENP-T, es esencial para la unión de condensina II a los centrómeros. Queda pendiente explorar si alguno de estos procesos guarda relación con la regulación de la cohesión centromérica por CENP-C.

Abbreviations

APC/C	Anaphase promoting complex/cyclosome
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Bub1	Budding uninhibited by benzimidazoles 1
CAP	Chromosome associated protein
CCAN	Constitutive Centromere Associated Network
CENP	Centromere protein
CPC	Chromosomal passenger complex
CSF	Cytostatic Factor
DAPI	4’6-diamidino-2-phenylindole
DDK	Dbf4/Drf1-dependent kinase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
HCG	Human chorionic gonadotropin
HJURP	Holliday junction recognising protein
IgG	Immunoglobulin G
INCENP	Inner centromere protein
KMN	KNL1/Mis12 complex/Ndc80 complex
min	minutes
Mps1	Monopolar spindle 1
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
Plk1	Polo-like Kinase 1
PMSG	Pregnant mare’s serum gonadotropin
PP2A	Protein phosphatase 2A
RbAp48	Retinoblastoma protein associated protein 48
SA	Stromal antigen, cohesin subunit
SAC	Spindle assembly checkpoint
Scc	Sister chromatid cohesion protein

Sgo	Shugoshin
siRNA	Small interfering ribonucleic acid
SMC	Structural Maintenance of Chromosomes
TBS	Tris buffered saline

1. Introduction

1. Introduction

In order for multicellular organisms to develop and maintain their health the process of cell division is essential. Chromosomes must be divided equally so each daughter cell has the exact same genetic material. When chromosome segregation does not occur correctly daughter cells can end up with damaged, too many or too few chromosomes. When a cell has the incorrect number of chromosomes this is called aneuploidy, this condition is associated with many developmental disorders and cancers (Pfau and Amon, 2012). The cell has a great number of mechanisms to ensure faithful chromosome segregation and avoid aneuploidy. In order for a cell to divide into two identical daughter cells first of all DNA is replicated during interphase. When the cell is ready to divide it enters a process called mitosis in which the chromosomes condense from an entangled chromatin mass into compact structures (prophase). At this stage for each chromosome there are two copies that are known as sister chromatids. The cell forms a spindle from microtubules that attach to the chromosomes aiding them to congregate at the midpoint of the cell (prometaphase/metaphase). The microtubules interact with a mitosis specific protein complex called the kinetochore. The kinetochore forms in a region of the chromosome called the centromere once the cell enters mitosis. Once all chromosomes are aligned at the midpoint and attached to microtubules the chromatids separate and are pulled to opposite ends of the cell (anaphase). Once the chromosomes are segragated the cell separates in to two cells (telophase).

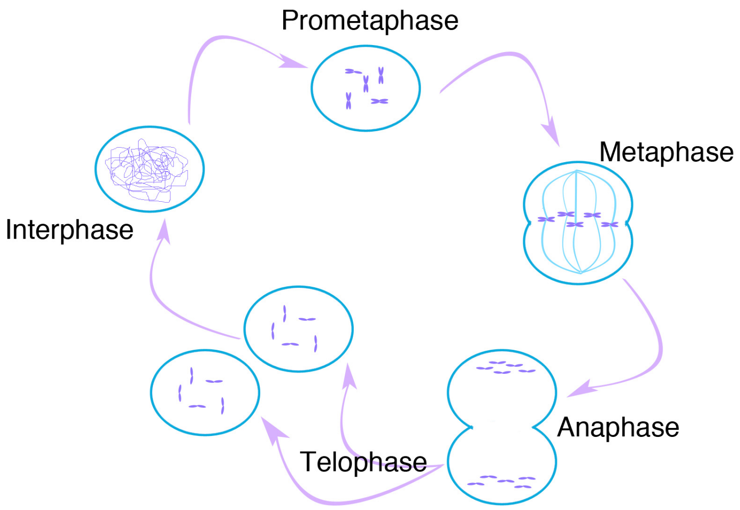


Figure I1: Stages of chromosome segregation. In interphase chromosomes are replicated, in prophase chromosomes compact, in metaphase chromosomes align at midpoint of cell, then in anaphase chromosomes separate towards the poles of the cell ready to be split into two daughter cells.

1.1 Centromere and kinetochore assembly

1.1.1 The constitutive centromere associated network

The centromere is located at the primary constriction site of the chromosome. It is epigenetically defined by the presence of a histone H3 variant known as centromere protein-A or CENP-A. CENP-A is interspersed with regular H3 nucleosomes to make up the centromeric chromatin (Blower et al., 2002). With the exception of budding yeast centromeres, CENP-A binding to DNA is not triggered by a specific sequence. It is common that centromeres contain regions of repetitive sequence however the sequence and length of repetitive region varies highly across species (Choo, 2001). Upon the centromeric CENP-A chromatin a collection of other CENP proteins bind and are known as the constitutive centromere associated network or CCAN. Identification of the first human centromere proteins, CENP-A, CENP-B and CENP-C was from immune serum from people that suffered with an autoimmune disease (Earnshaw and Rothfield, 1985). Later further centromere proteins were identified in yeast, the yeast COMA complex is the equivalent of the human CENP-O-P-Q-U complex (De Wulf et al., 2003). The homologous human proteins were later found along with several other novel CENP proteins, CENP-K,L,M,N,O,P,Q,R,S,T,U had now been described (Obuse et al., 2004, Okada et al., 2006, Foltz et al., 2006, Izuta et al., 2006). Identification of CENP-W (Hori et al., 2008) and CENP-X (Amano et al., 2009) later followed. In drosophila only CENP-C is present, homologues of other CCAN proteins have not been identified. It has been possible to characterise the CCAN into a range of subcomplexes. They consist of CENP-C, CENP-L-N, CENP-T-W-S-X, CENP-H-I-K-M and CENP-O-P-Q-R-U (Okada et al., 2006, Basilico et al., 2014, Perpelescu and Fukagawa, 2011, Westhorpe and Straight, 2015). These CCAN subcomplexes constitute the inner kinetochore.

The CCAN is present throughout the cell cycle and dependent on the presence of CENP-A although it has been shown that the CCAN can persist when CENP-A levels are reduced (Liu et al., 2006, Fachinetti et al., 2013). CENP-C is known to directly recognise and interact with CENP-A nucleosomes (Carroll et al., 2010). CENP-N is also thought to recognise CENP-A nucleosomes (Carroll et al., 2009). While it is suggested that CENP-T recognises the adjacent H3 nucleosomes in the centromeric chromatin (Hori et al., 2008, Ribeiro et al., 2010). The dynamics of the CENPs loading on to chromatin and the cell cycle stage at which they are loaded has also been studied. CENP-A loads on to chromatin in human cells during the transition from mitosis to G1 and is very stably bound (Bodor et al., 2013). CENP-T-W binds in S phase and G2 (Prendergast et al., 2011). All the CENP proteins appear to be stably bound during mitosis (Hemmerich et al., 2008). It has been seen that there is more CENP-N at centromeres in interphase than mitosis and it is likely new CENP-N is loaded then (McClelland et al., 2007, Hellwig et al., 2011). Whether CENP-T-W-S-X is always a complex or is two separate complexes that associate with each other is still under contention. In chicken cells depletion of CENP-S or CENP-X did not produce an apparent reduction in other CCAN proteins like CENP-T depletion does suggesting that CENP-T is upstream (Amano et al., 2009). How the CCAN subcomplexes interact with and depend on each other plus whether they have specific functions are still ongoing questions.

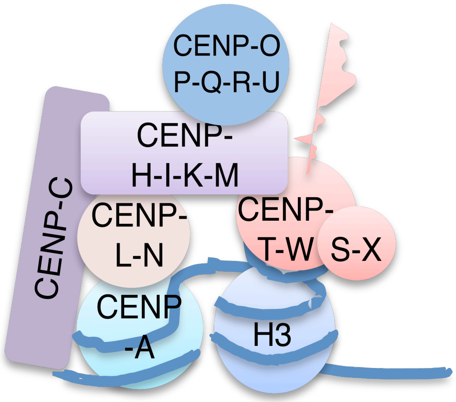


Figure I2: The constitutive centromere associated network. Basic representation of the defined CCAN subcomplexes.

1.1.2 CCAN subcomplex hierarchy

There have been many studies done on the interdependencies between the different members of the CCAN complexes. There is some disparity across this work; variations in results across model systems and methods. Whilst it is clear that CENP-C has importance in either recruitment or maintenance of the other CCAN complexes whether or not they are all recruited downstream of CENP-C is under debate. The method most commonly used to study this up to now is RNA interference to deplete proteins in human cells. This means a gradual depletion of the protein over several cell cycles. There is also commonly variation in efficiency of depletion with this system. In one study in human cells CENP-C siRNA disrupts CENP-H-I-K-M and CENP-T recruitment to centromeres (Carroll et al., 2010). While siRNA depletion of CENP-C in another study did not cause loss of CENP-T but did cause loss of CENP-H at centromeres (Gascoigne et al., 2011). Some results have proven to be consistent in this system. In several studies depletion of CENP-N with siRNA has been shown to cause a decrease in binding of the CENP-H-I-K-M subcomplex (Foltz et al., 2006, McClelland et al., 2007, Carroll et al., 2009). CENP-N depletion has also been reported to cause a decrease in CENP-C levels at centromeres in human cells (Carroll et al., 2009).

Another system exploited for studying the CCAN hierarchy is genetic knock out. This can give the opportunity to study the effect on centromeres assembled in the absence of the deleted protein. Interestingly in CENP-C knock out chicken cells CENP-T, CENP-H, CENP-O and CENP-S complexes can still be seen at centromeres (Hori et al., 2008). The other possibility is an inducible knock out which is generally more efficient than siRNA but still results in a gradual depletion over several cell cycles from an already established centromere. Experiments using inducible genetic knockout of CENP-A in human cells showed that CENP-A levels have to be almost completely abolished for loss of the other CENPs. Levels of CENP-C and CENP-N have a more linear correlation with the amount of CENP-A than CENP-I and CENP-T. CENP-T and CENP-I are able to maintain higher levels even with reduced CENP-C and CENP-N (Fachinetti et al., 2013). This indicated the possibility that CENP-H-I-K-M and CENP-T are not dependent on the presence of CENP-C or CENP-N in human cells. However, very recently inducible genetic knockouts of CENP-N, CENP-I or CENP-T showed that complete loss of any of those three CCAN members resulted in loss of both of the other two at centromeres (McKinley et al., 2015). The loss of CENP-H-I-K-M with depletion of CENP-T has also been seen in other systems. siRNA depletion of CENP-T in human cells caused reduction of CENP-H and CENP-M (Gascoigne et al., 2011, Foltz et al., 2006). While chicken CENP-T knock out cells still have CENP-C but lose CENP-H (Hori et al., 2008). Whether CENP-T depletion affected CENP-L-N localisation was not previously clear.

The CENP-O-P-Q-R-U appears to be downstream of the other subcomplexes. In human cells CENP-U depletion did not affect CENP-H, CENP-M or CENP-N only CENP-O and CENP-P (Foltz et al., 2006). While siRNA against CENP-O also did not cause loss of CENP-N or CENP-H (McClelland et al., 2007). CENP-O recruitment has been seen to be dependent on CENP-N, CENP-H and CENP-T (McClelland et al., 2007, Hori et al., 2008).

Differences in dependencies have also been seen depending on cell cycle state. In chicken cells CENP-C requires CENP-H-I-K-M in order to localise to centromeres in interphase but not mitosis (Fukagawa et al., 2001, Kwon et al., 2007). This was also confirmed recently in human cells when CENP-N or CENP-I were rapidly degraded specifically in interphase CENP-C levels were reduced at centromeres. Whilst if they were degraded in cells arrested in mitosis levels of CENP-C were not affected (McKinley et al., 2015). As summarized in table I1 there is great variation in results creating complexity in understanding the CCAN hierarchy. Key questions that need to be resolved include:

1. Whether CENP-C is absolutely required for binding of the other CCAN members
2. Whether the CENP-T-W-S-X, CENP-H-I-K-M and CENP-L-N complexes are completely dependent on each other for centromere binding

Table I1: summary of observed interdependencies in the CCAN

depleted protein	effect				
	CENP-C	CENP-T-W-S-X	CENP-L-N	CENP-H-I-K-M	CENP-O-P-Q-R-U
CENP-C	-	**human** - varying degrees of dependency from no decrease to complete loss at centromeres **chicken** - remains at centromeres	**fission yeast** - reduced at centromeres	**human** - lost at centromeres **chicken** - remains at centromeres	**chicken** - reduced but present at centromeres
CENP-T-W-S-X	**human** - no dependency **chicken** - no dependency	-	**human** – lost at centromeres	**human** - reduced recruitment **chicken** - lost	**chicken** – lost at centromeres
CENP-L-N	**human** - decrease	**human** – lost at centromeres	-	**human** - reduced or lost	**human** – lost at centromeres
CENP-H-I-K-M	**chicken** - decreased in interphase but not mitosis	**human** – lost at centromeres	**human** – lost at centromeres	-	**human** – lost at centromeres
CENP-O-P-Q-R-U	**human** - unaffected		**human** - unaffected	**human** - unaffected	-

Reference not previously stated in text (Tanaka et al., 2009)

1.1.3 Functions of the CCAN

The CCAN has been the focus of many studies since its discovery one goal is to characterise the functions of the CCAN proteins. Various members have been shown to play a role in CENP-A loading. In *Xenopus* CENP-C depletion has been shown to affect CENP-A loading (Moree et al., 2011, Krizaic et al., 2015). CENP-N has also been associated with regulation of CENP-A levels (Carroll et al., 2009). While in yeast and chicken the CENP-H-I-K-M complex has been implicated in incorporation of new CENP-A (Okada et al., 2006, Takahashi et al., 2000).

The main identified function of the CCAN is that it provides the interface for outer kinetochore proteins, and consequently spindle microtubules, to bind during mitosis (Figure I3). The outer kinetochore KMN network consists of three protein complexes; Mis12, Ndc80 and KNL1. The KMN network binds microtubules (Cheeseman et al., 2004, Cheeseman et al., 2006) and recruits other kinetochore proteins including spindle assembly checkpoint (SAC) proteins. It is known that CENP-C interacts directly with the Mis12 complex via its N-terminal while the N-terminal of CENP-T has a direct interaction with the Ndc80 complex (Gascoigne et al., 2011, Nishino et al., 2013, Schleiffer et al., 2012). The CENP-H-I-K-M complex was also shown to be important for KNL1 recruitment (Cheeseman et al., 2008). In drosophila where other CENP proteins are not present CENP-C can recruit the whole KMN (Przewloka et al., 2011). It has also been proposed that CENP-H could be important in regulating microtubule plus-end dynamics (Amaro et al., 2010). Another suggested function for the CCAN is in tension resistance (Suzuki et al., 2014). Whether CCAN proteins have other specific functions remains to be identified.

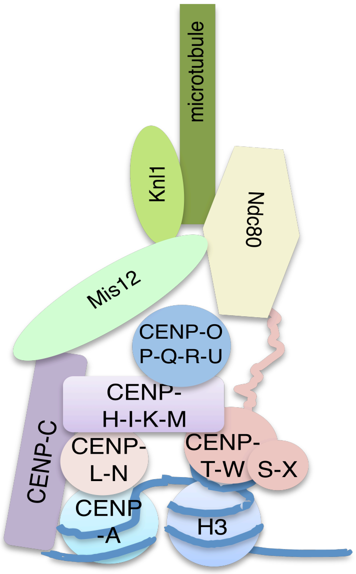


Figure I3: The CCAN acts as an interface for KMN network binding. CENP-C interacts with the Mis12 complex. CENP-T interacts with the Ndc80 complex.

1.2 Centromeric cohesion

1.2.1 Cohesion

The cohesin protein complex is made up of four main subunits: the structural maintenance of chromosomes subunits SMC1 and SMC3, Rad21 and the stromal antigen SA subunit. It can also interact with other regulating proteins Pds5, sororin and Wapl. Cohesin binds to chromatin during early G1, the pre replication stage of interphase. The cohesin binding proteins Pds5 and Wapl are responsible for the unloading of cohesin during this time, meaning the binding of cohesin to chromatin is very dynamic. During replication cohesion of sister chromatids is established when cohesin becomes acetylated and sororin displaces Wapl preventing it from dissociating cohesin from chromatin. (Nishiyama et al., 2010) Once the cell enters prophase various phosphorylations occur. The SA subunit of cohesin is phosphorylated by Plk1. Sororin is phosphorylated by Aurora B and Cdk1 causing it to dissociate from cohesin. This causes the majority of cohesin to be removed from the chromosome arms. The proportion of cohesin that remains is mainly concentrated at the centromere region. Once the cell enters anaphase the anaphase promoting complex/cyclosome (APC/C) promotoes degradation of securing. Separase is no longer sequestered by securin and becomes free to cleave the Rad21 cohesin subunit (Uhlmann et al., 1999). The remaining cohesin is released from chromosomes and they are able to segregate (Figure I4).

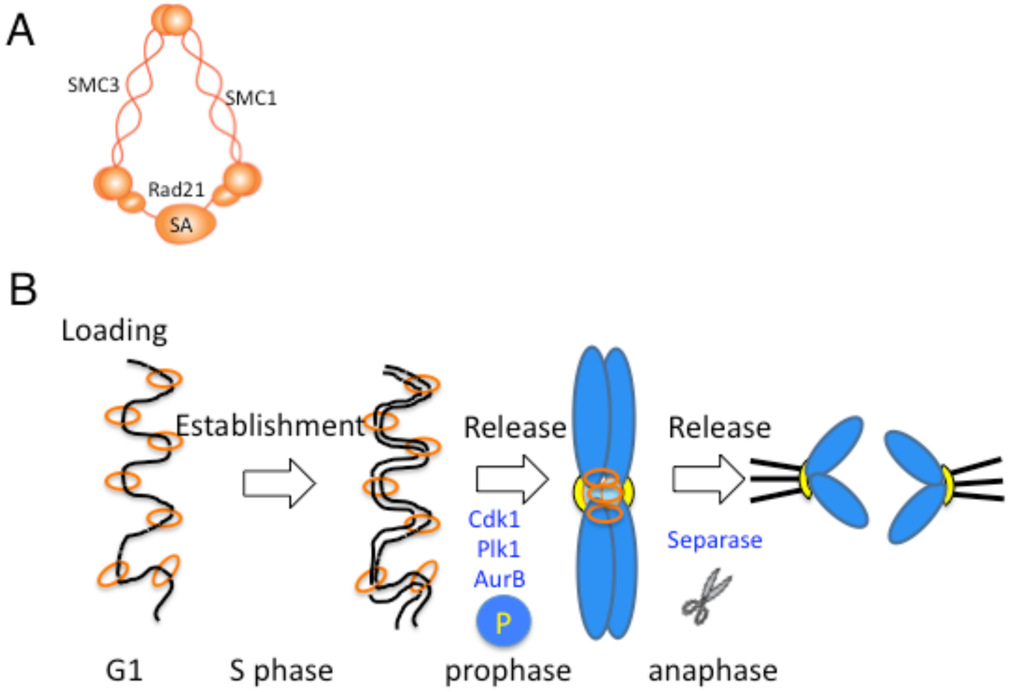


Figure I4: Cohesin loading and release. (A) Cohesin complex consists of 2 SMC subunits, both of which interact with the Rad21 kleisin subunit which binds the stromal antigen SA subunit. **(B)** cohesin loads during G1 and is established during S-phase. On entry to mitosis most of cohesin removed from chromosomes due to phosphorylations. The remaining cohesin is removed by spearase on entry into anaphase.

1.2.2 Centromeric cohesion and Shugoshin 1

The maintenance of the population of cohesin at centromeres until anaphase is essential for faithful chromosome segregation. Many studies have been done to identify the proteins important for maintenance of centromeric cohesion. It was known that the yeast protein shugoshin (Sgo) and its homologue in drosophila MEI-S332 were important for centromeric cohesion in meiosis I. It was then later identified that the shugoshin homologues in *Xenopus laevis* and human were important for sister chromatid cohesion in mitosis (Salic et al., 2004, McGuinness et al., 2005). It then went on to be found that cohesin at centromeres is protected by shugoshin 1 (Sgo1) in conjunction with protein phosphatase 2A (PP2A). This buildup of shugoshin 1 protects cohesin and sororin from phosphorylation and therefore prevents dissociation of cohesin at this area. Sgo1's partner PP2A is thought to counteract the Plk1 phosphorylations of cohesin (Kitajima et al., 2006, Riedel et al., 2006, Tang et al., 2006).

Bub1 is essential for Sgo1 recruitment to the centromere (Tang et al., 2004, Kitajima et al., 2005). It phosphorylates histone H2A on serine 121 in yeast and threonine 120 in humans and *Xenopus laevis* (Kawashima et al., 2010). Bub1 is recruited to kinetochores when the kinetochore protein KNL1 is phosphorylated by the kinase Mps1 triggering accumulation of Bub3 and therefore Bub1 (Krenn et al., 2012, Yamagishi et al., 2012, Vleugel et al., 2013). The chromosomal passenger complex (CPC), which consists of four subunits including the kinase Aurora B, has been shown to be required for recruitment of both Bub1 and Sgo1. It has also been shown that the CPC plays a role in the recruitment of Mps1 to kinetochores (Saurin et al., 2011). Although Bub1 recruitment has been studied in the context of the spindle assembly checkpoint (SAC) and Sgo1 recruitment can be inferred from this, nobody has specifically investigated requirements for Sgo1. The heterochromatin protein HP1 has also been implicated in recruitment of Sgo1 to the centromere (Yamagishi et al., 2008).

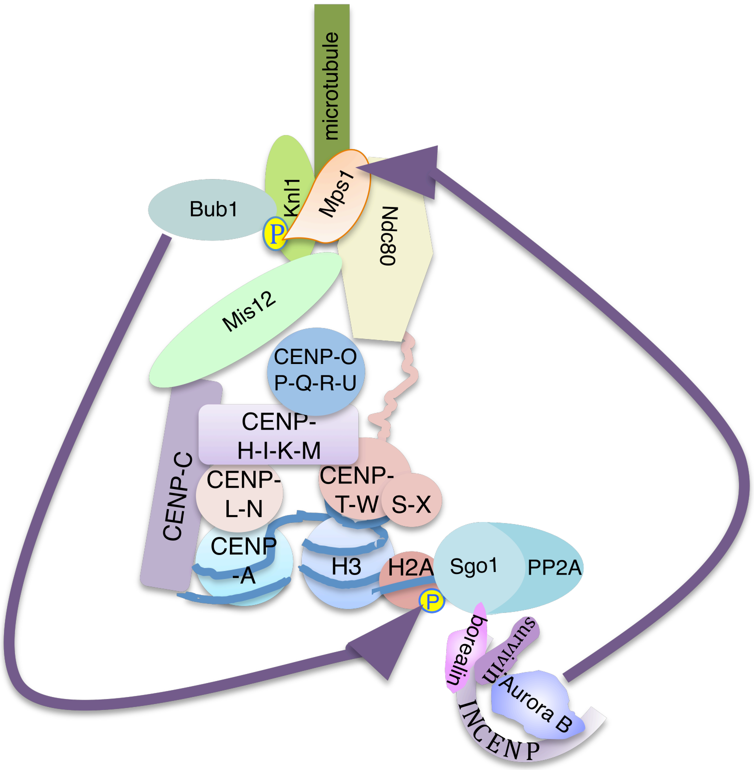


Figure I5: Sgo1 recruitment. Mps1 phosphorylates KNL1 allowing Bub1 accumulation. Bub1 phosphorylates histone H2A allowing Sgo1 accumulation. The CPC is also required for Bub1 and Sgo1 accumulation possibly through its role in Mps1 recruitment.

1.2.3 Interplay of kinetochore assembly with centromeric cohesion

It is clear that the centromere and kinetochore regions have a very complex interplay of proteins. Which members are necessary for Sgo1 recruitment and therefore centromeric cohesion remains unclear. There have been limited clues on the role of the CCAN in Sgo1 recruitment. In yeast the CENP-L homologue Iml3 and CENP-N homologue Chl4 were identified along with Sgo1 as required for centromeric cohesion (Marston et al., 2004). More recently it was also seen in yeast that CENP-N and Sgo1 can directly interact in vitro (Hinshaw and Harrison, 2013). However, GFP-CENP-N does not localise to engineered human ectopic kinetochores while Sgo1 could be recruited to a percentage (Gascoigne et al., 2011). Suggesting CENP-N is not required for Sgo1 recruitment in humans. It has been suggested that the CCAN is important for correct centromeric cohesion. The CCAN equivalent in yeast the Ctf19 complex has been implicated in establishment of cohesion. It has been proposed that is required for increased loading of cohesin on to the pericentromeric chromatin (Fernius and Marston, 2009). This is essential for faithful chromosomes segregation in yeast.

1. 3 Centromeric Condensin II

1.3.1 Condensins

Apart from cohesin there are another class of SMC proteins called condensins. In most eukaryotes there are two family members condensin I and condensin II. Condensin II accumulates at centromeres as well as being spread along the chromosomes arms in humans, xenopus and drosophila. The ratio of condensin II and condensin I differs between species. Some species including yeasts have lost condensin II while in C.Elegans it is the most prevalent condensin. How and why condensin II functions differently across species remains an interesting question for both chromosome dynamics and evolution. In *Xenopus laevis* egg extracts condensin I is more prevalent then condensin II it has been suggested there is five times more condensin I. In HeLA cells the abundance appears equal (Ono et al., 2003). Nonetheless an accumulation of condensin II is seen at centromeres in *Xenopus laevis* egg extracts as in human cells.

Prevalence of condensin proteins in different species

	Condensin I	Condensin II
human	similar to II	similar to I
Xenopus	most abundant	5 times less than condensin I
C.Elegans	Low abundancy	most abundant
yeast	only condensin	not present

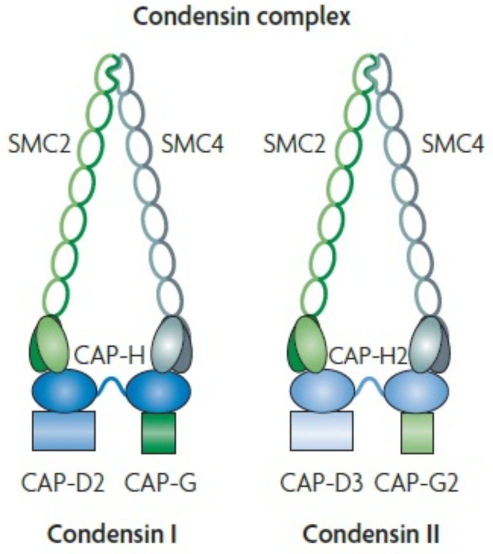


Figure I6: Condensins I & II have varying prevalence in different species. Diagram of condensin complexes taken from Wood et al., 2010.

1.3.2 Function of condensin II at the centromere

Condensin II function seems to be divergent across species; in fact during evolution yeasts have even lost condensin II. In the plant Arabidopsis thaliana condensin II is dispensable for chromosome segregation but has a role in alleviating DNA damage (Sakamoto et al., 2011). Condensin II is present in the nucleus while condensin I is sequestered to the cytoplasm until nuclear envelope breakdown in most organisms (Ono et al., 2004, Hirota et al., 2004). It is becoming increasingly clear that condensin II has functions in genome organisation in interphase. It has been suggested that condensin II may function at centromeres in meiosis. However, accumulation of condensin II can be seen at mitotic centromeres in several species including humans, *Xenopus laevis* and *Drosophila*.

The function of condensin II enrichment at centromeres during mitosis is not fully understood. Our lab previously described a role for it in CENP-A deposition in *Xenopus laevis* (Bernad et al., 2011). Depletion of an SMC subunit of condensin that is present in both condensin I and II has also been described to cause a defect in CENP-A deposition in human cells (Samoshkin et al., 2009). Up to now condensin II has not been shown to specifically cause a defect in human cells. In the algae C.merolae condensin II is enriched at centromeres and may function in sister chromatid resolution but is not essential for faithful chromosome segregation (Fujiwara et al., 2013). Condensin II has also been implicated in sister centromere resolution in the nematode worm C.elegans (Moore et al., 2005). In both cases defects were seen when microtubule function was compromised. Several studies suggest that condensin I is required for structural integrity of centromeres but condensin II has not been implicated in this (Oliveira et al., 2005, Gerlich et al., 2006). Links between condensin and cohesion have been suggested (Lam et al., 2006). It seems like sister chromatid cohesion may be dependent on be a fine balance between cohesin and condensins. In human cells it has been shown that condensin I loading at entry to mitosis is important for removal of cohesin (Hirota et al., 2004). While more recently it has been shown that condensin II can counteract cohesion in S-phase (Ono et al., 2013).

1.3.3 Condensin II recruitment to the centromere

Up to now a definitive pathway for the recruitment of condensin II to centromeres has not been elucidated. In human cells Aurora B is known to be important for accumulation of condensin II at centromeres but not for binding to chromosome arms (Ono et al., 2004). In C.Elegans condensin II is the main form of condensin and Aurora B is required for it to be recruited to chromosomes (Hagstrom et al., 2002) although this has been disputed (Maddox et al., 2006). In HeLa it has been shown that Cdk1 phosphorylation of the condensin II subunit CAP-D3 is essential for it to be recruited to chromatin. This in turn is required for Plk1 phosphorylation of condensin II which is required for correct function (Abe et al., 2011). In HeLa cells phosphorylation of condensin II subunit CAP-H2 by the kinase Mps1 has shown to be important for correct recruitment of condensin II to chromatin (Kagami et al., 2014). Protein phosphatase 2A (PP2A) is required for condensin II recruitment to chromosome arms in Xenopus egg extracts and human cells (Takemoto et al., 2009). In human cells knockdown of Sgo2 caused a decrease in accumulation of condensin II at centromeres (Takemoto et al., 2009). In yeast where there is only one condensin complex Sgo1 has been implicated in the recruitment of condensin to centromeres (Peplowska et al., 2014, Nerusheva et al., 2014, Verzijlbergen et al., 2014). Little is known about the requirements of the CCAN proteins for condensin II recruitment to centromeres. It has been reported in human cells that siRNA depletion of CENP-I causes a defect in CAP-H2 accumulation at centromeres (Nakazawa et al., 2008). CENP-C is required for recruitment of condensin II to centromeres in C.elegans (Moore et al., 2005).

Table I2 Summary of known recruitment factors for condensin II

	Human condensin II	**Xenopus condensin II**	**C.Elegans condensin II**	**Yeast condensin**
Aurora B	centromere		arms	
Cdk1	arms			arms
Mps1	arms			
PP2A	arms	arms		
Sgo2	centromere			n.p
Sgo1				centromere
CENP-I	centromere		n.p	
CENP-C			centromeres	

n.p signifies this protein is not present in the organism

1.4 Xenopus System

Xenopus Laevis is a common model system used for cell and developmental biology research. More than 30 years ago it was shown that eggs from Xenopus laevis could be crushed to produce a protein extract that would recapitulate processes of mitosis in vitro when sperm nuclei were added; nuclear envelope breakdown, chromosome condensation and spindle formation (Lohka and Masui, 1983, Lohka and Maller, 1985). Since this time the system has been widely used for cell biology studies. When eggs are collected from the frogs they are arrested in metaphase of meiosis II. Meiosis II is essentially the same process as mitosis, separation of sister chromatids in to two new daughter cells. Eggs are arrested in this state by cytostatic factor (CSF) therefore extracts made from the eggs are deemed CSF extracts. To make the extracts, eggs are crushed by centrifugation and the cytoplasmic fraction is removed. CSF extracts can be converted to interphase by adding calcium, which causes the CSF to be degraded. Once in interphase extracts can support DNA replication and nuclear membrane formation. It is also possible to generate interphase extracts by adding calcium to the eggs before preparation of the extract. Interphase extracts can then be cycled in to mitosis again by adding either more CSF extract or recombinant cyclin B protein. When looking at mitotic chromosomes assembled in the extract by immunofluorescence they appear as an entangled mass not as separated chromosomes like is seen in human cells. Cycled mitotic chromosomes have paired sister centromeres whilst the unreplicated CSF chromosomes have just a single centromere (Figure I7).

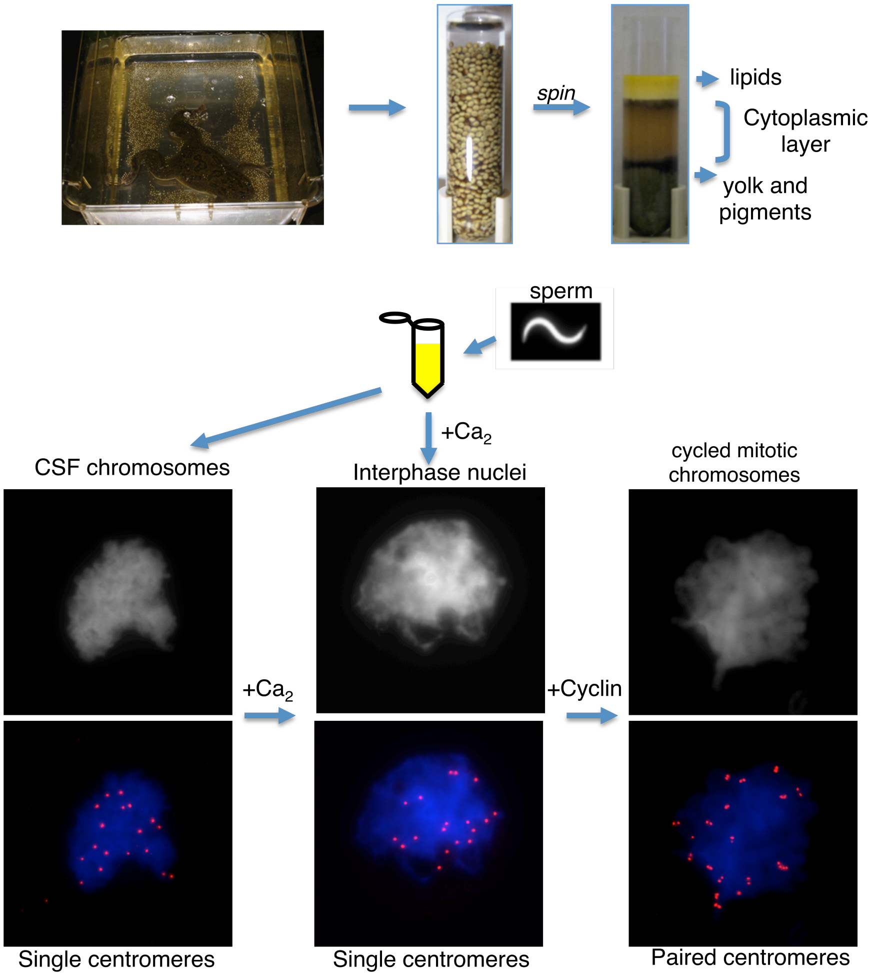


Figure I7: Xenopus egg extract preparation and chromosome assembly. Eggs arrested in metaphase II by Cytostatic factor (CSF) are crushed and the cytoplasmic fractions. Adding sperm to CSF extract assembles unreplicated mitotic chromosomes. Adding calcium to extract causes break down of CSF and entry into interphase. Addition of CSF extract or Cyclin B to interphase extracts induces entry back into mitosis.

There are many advantages of using the *Xenopus laevis* egg extract cell-free system. DNA from *Xenopus laevis* sperm does not contain most chromatin binding proteins (Philpott and Leno, 1992). Therefore, we can assemble de novo centromeres on a naïve template containing only CENP-A at the centromere region (Zeitlin et al., 2005, Milks et al., 2009). By using the *Xenopus* system to address recruitment we can bypass some of the caveats of siRNA studies in cells. We deplete proteins before any assembly and can look at the effect across one cell cycle, with gradual depletion across many cell cycles errors can accumulate and compensation can occur. One other benefit is that the extract is completely synchronous. For rescue experiments it is possible to add either recombinantly expressed protein or mRNA that the extract will translate to protein. Although the *Xenopus laevis* egg extract system is great for recruitment studies it is limited in functional assays. As it does not pass through to anaphase in our conditions it is not possible to look for chromosome segregation defects for example.

When sperm is added to the CSF extracts unreplicated mitotic chromosomes assemble, by adding calcium to the extract this can cycle the chromosomes into interphase where they will replicate. By then adding cyclin or more CSF extract you can cycle the extract into mitosis and have replicated mitotic chromosomes. CSF kinetochores have been described as being less robust with a decreased binding of many kinetochore proteins compared to 'cycled' replicated mitotic chromosomes (Maresca and Heald, 2006). This difference can have functional consequences, for example a difference in the effect of Bub1 depletion on other outer kinetochore proteins in CSF and replicated mitotic chromosomes has been seen (Boyarchuk et al., 2007). A lot of studies in *Xenopus laevis* egg extracts have been done purely in CSF extracts (Vigneron et al., 2004, McCleland et al., 2003). Therefore, it is important in interpreting results from these studies that we understand the differences in these centromeres compared to the cycled centromeres.

2. Objectives

2. Objectives

The centromere is characterised by the prescence of the histone H3 variant centromere protein A (CENP-A). Upon CENP-A binds a large number of other CENP proteins that make up the constitutive centromere associated network (CCAN). These proteins have complex interdependencies and functions that can vary between systems. The centromere is the region were a population of cohesin remains during mitosis and maintains cohesion of the sister chromatids until they need to separate at the end of mitosis. This population of cohesin is protected by the protein shugoshin 1 (Sgo1), but how Sgo1 accumulates specifically at this region is not fully defined. Condensin II also accumulates at centromeres in mitosis but its function and how it is recruited are unclear. The Xenopus egg extract cell-free system is ideal for studying recruitment. Therefore, the objectives of this thesis were:

1. Characterise the assembly of the CCAN in different cell cycle stages.

2. Characterise the hierarchy of interactions among CCAN subcomplexes.

3. Define the requirements of centromere proteins in Sgo1 recruitment and protection of cohesion at centromeres.

4. Investigate which known centromere proteins are important in condensin II centromeric accumulation.

3. Materials and Methods

3. Materials and Methods

3.1 Antibody Generation

We generated new antibodies for this study. To produce an antibody against phosphorylated threonine 120 of Xenopus laevis histone H2A rabbits were injected with with the phosphopeptide peptide CLLPKK(pT)ESAKS (Innovagen, SE). For the antibodies against Xenopus Laevis CENP-N and CENP-K insoluble recombinant 6xHis tagged proteins were expressed in bacteria and then purified using Nickel beads. Proteins were partially resolubilised by using increasing concentrations of urea. The proteins were then injected into rabbits (Innovagen, SE) and the antibodies were purified from the rabbit serum. Other antibodies used in this study are detailed in table M1.

Table M1. Antibodies used in the study.

Antibody	**Provider/Reference**
xCENP-A	Rivera & Losada, 2009
xCENP-C	Krizaic *et al*, 2015
xCENP-T	Krizaic *et al*, 2015
xCENP-N	Generated for this study
xCENP-K	Generated for this study
xMis12	Emanuele *et al*, 2005
xNdc80	Emanuele *et al*, 2005
xMps1	Morin *et al*, 2012
xBub1 monoclonal	Rivera & Losada, 2009
xBub1	Rivera & Losada, 2009
xSgo1 monoclonal	Rivera & Losada, 2009
xSgo1	Rivera & Losada, 2009
xpH2a	Generated for this study
B4 (embryonic histone H1)	Bernad *et al*, 2011
xRbAp48	Bernad *et al*, 2011
xSMC3	Losada *et al*, 1998
xSMC1	Losada *et al*, 1998
xRad21	Losada *et al*, 1998
xAuroraB	MacCallum *et al*, 2002
xINCENP	MacCallum *et al*, 2002
myc	CNIO monoclonal antibody unit
xCAP-H2	Losada *et al*, 1997
xCAP-D3	Bernad *et al*, 2011
xCAP-G2	Losada *et al*, 1997
xCAP-G	Losada *et al*, 1997
hSgo1	Serrano *et al*, 2009
ACA	Antibodies Incorporated

3.2 Preparation of Xenopus egg extracts

Eggs arrested in meiosis II are obtained by hormonation of female *Xenopus laevis* frogs. They are hormoned on day one and day three with gonadotropin from pregnant mare serum (PMSG). Then between three and five days later they are hormoned with human chorionic gonadotropin (HCG), 24 hours before collection of the eggs. CSF extracts are prepared from the collected eggs using the following protocol. Eggs are washed with MMR buffer (100mM NaCl, 2mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 0.1mM EDTA, 5mM HEPES, pH7.8). Eggs are then incubated with dejellying solution (2% cysteine) for up to 10 minutes. They are then washed with XBE2 (100mM NaCl, 0.1mM CaCl2, 1mM MgCl2, 5mM EGTA, 50mM sucrose, 10mM HEPES, pH7.7). Using a glass pipette, with a wide opening, eggs are transferred to 5ml ultracentrifuge tubes, packed by spinning at 1500rpm for 1min at 16°C and excess buffer removed. They are then crushed by spinning at 10000rpm for 15mins at 16°C. The cytoplasmic fraction is removed by puncturing the side of the tube with a needle and syringe. Then leupeptin/pepstatin/chymostatin protease inhibitors at 10μg/ml, 10μg/ml cytochalasin B and energy mix (1mM ATP, 1mM MgCl2, 10mM creatine phosphate, 50μg/ml creatine kinase) are added. The extracts are then spun at 10000rpm for 10mins at 4°C and the cytoplasmic fraction removed. This is known as a low speed extract and is used for all experiments in which chromosomes are assembled. If low speed extract is spun for 2 hours at 50000rpm more lipids and membranes can be removed and a high speed extract is obtained. This extract is used for biochemical analysis only.

The CSF extract can be converted to interphase by adding first 100μg/ml cycloheximide for 5 minutes at room temperature followed by 0.7mM $CaCl_2$ for 30 minutes at 22°C. To prepare interphase extracts the procedure is very similar. Before the XBE2 washes, eggs are incubated for 10 minutes with calcium ionophore (sigma) in MMR then the buffer is changed to only MMR for 10 further minutes. From this point on the procedure is the same but the eggs are always kept at 4°C including the packing and crushing spins.

3.3 Assembly of chromosomes in the extract

To obtain unreplicated mitotic chromosomes, sperm (~1000 nuclei/µl) is incubated in CSF extracts for 90 min at 22°C. To obtain replicated chromosomes, sperm is first incubated in interphase extracts for 90 min at 22°C. Then, an equal volume of CSF extract or 100 nM sea urchin cyclin B (purified from a plasmid kindly provided by T. Hirano) is added to the assembly mixtures. These are incubated for additional 90 min at 22°C before processing them for analysis by immunofluorescence. Chromatin assembly reactions for immunoblot analysis were carried out in the same way but increasing sperm concentration to 2000 nuclei/µl and always using cyclin B for driving entry in mitosis. To isolate chromatin for western blotting, the assembly mixture is resuspended in 10 volumes of 0.25% TX-100 in XBE2 and left on ice for 10 minutes. It is then added on to a 1ml 30% sucrose cushion and spun in a swing out microcentrifuge at 9000rpm for 15mins at 4°C. The cushion is then washed four times with XBE2 before removal of the majoriy of the cushion. 60*µ*l of the cushion is left and the tubes are spun again at full speed in a fixed angle rotor microcentrifuge at 4°C. With the chromatin attached to the side of the tube the rest of the cushion can be carefully removed with gel loading tips and the chromatin is resuspended in 10*µ*l of 1XSDS sample buffer.

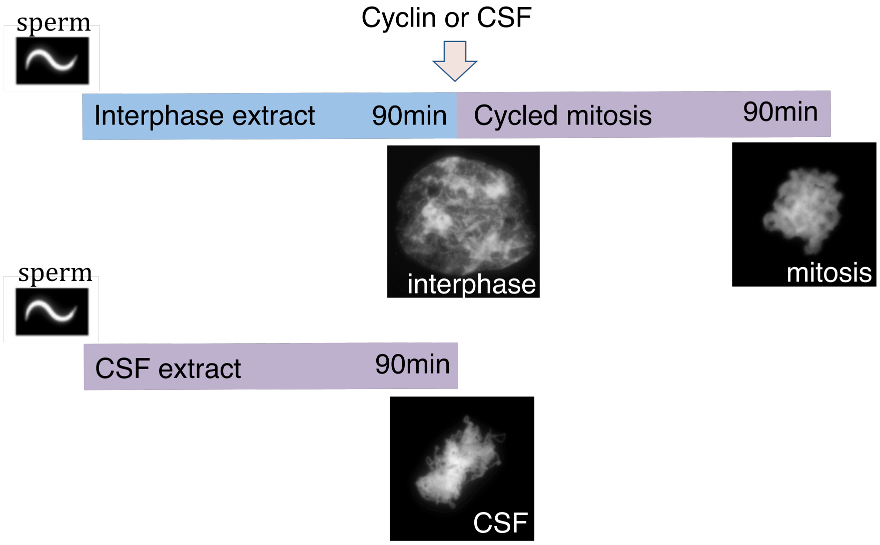


Figure M1. Assembly of chromosomes or nuclei in Xenopus egg extract. Demembranated sperm is added to either interphase or CSF extracts. After 90 minutes of incubation CSF chromosomes or interphase nuclei can be fixed. For cycled mitotic extracts cyclin B must be added to the interphase extracts for a further 90 minutes before fixation.

3.4 Immunodepletion and protein addition

Depletions were done using protein A pureproteome magnetic beads (Millipore) or protein A dynabeads (Life technologies). To deplete 100μl of extract required 2 rounds of depletion with 50μl of beads bound to 30μg of antibody. The antibody was incubated with the beads for 1 hour at room temperature with rotations, followed by rotation at 4°C overnight. For CPC depletion a combination of both INCENP and aurora B antibodies was used. For depletion of cohesin a combination of SMC1 and SMC3 antibodies was used. In order to get efficient depletion of Sgo1 it was necessary to use 2 rounds of depletion with affiprep beads (BioRad). For some immunofluorescence experiments beads were crossliniked to the antibody using dimethylpimelidimate dissolved in sodium borate for 30 minutes, then washed and incubated with ethanolamine.

For addition of Bub1 targeting protein first the fragment of Xenopus CENP-C coding for amino acids 712–1400 was cloned into pCS2+myc vector (cenC). The fragment was amplified from full length CENP-C in pCS2+myc vector that was provided by AF Straight (Moree et al., 2011). The kinase domain of Xenopus Bub1 (amino acids 490–1136) was then added (cenBub1). To generate myc-CENP-N and myc-CENP-L full length cDNAs were inserted into the pCS2+myc vector. Corresponding myc-tagged proteins were produced with TNT Quick Coupled Transcription/Translation system (Promega).

3.5 Immunofluoresence

For fixation of *Xenopus laevis* chromosomes 10 volumes of 2% Paraformaldehyde with 0.25%TX-100 in XBE2 is used for 10 minutes at room temperature. They are then spun through a 5ml 30% glycerol in XBE2 cushion at 6500rpm for 15mins at 4°C onto coverslips. Coverslips are washed with washing solution (1XTBS/0.1% TX-100) and then blocked overnight with blocking solution (3% BSA/1XTBS/0.05%TWEEN). Coverslips are incubated with primary antibody in blocking solution for 2-3hours. At a concentration of 2μg/ml for CENP-A, CENP-C, CENP-T and pH2A, 5μg/ml for Sgo1, Bub1, CAP-H2, CAP-G2, CENP-N, CENP-K and Mis12, 10μg/ml for SMC3 or 1:100 dilutions of myc, Ndc80 and Mps1. Secondary antibodies were diluted 1:200 and incubated with the coverslips for 1-2 hours. For labelling centromeres when the primary antibody was raised in rabbit, after incubation with the secondary antibody we blocked for 1hour with 3%BSA containing 200μg/ml rabbit IgG. Then incubated with CENP-A or CENP-C labelled with fluorescent tag 594 or CENP-A conjugated to biotin (5μg/ml). After the biotin labelled CENP-A incubation coverslips are washed and then incubated with strepdavidinCy3 (1:500 dilution) for 1 hour. For xINCENP or xCAP-G staining antibodies labelled with a 488 fluorophore were used.

For immunofluorescence of cells in the siCENP-C/siCENP-T experiments cells are given hypotonic treatment with 60mM KCl for 30 mins at room temperature before fixation. For the siCAP-D3 experiments to look at GFP-CENP-A cells are preextracted for 5 mins with CSK buffer (10mM Pipes, pH7, 0.1M NaCl, 0.3M sucrose, 3mM $MgCl_2$, 0.5% TX100, 0.5mM PMSF) on ice before fixation. Cells are then fixed with 2% PFA in PBS for 15 minutes at room temperature. Following this they are permeabilised for 5 min at room temperature with 0.2% Triton-X100 in PBS. They are then blocked for 1 hour with 3% BSA in PBS. Incubated with primary antibodies for 1-2 hours hSgo1 antibody is used at a concentration of 5μg/ml and the ACA antibody at 1:100 dilution. Coverslips were then incubated with secondary antibodies diluted 1:200 for 1-2 hours.

For both assembled Xenopus chromosomes and human cells the DNA is stained with DAPI in wash solution for 10 seconds, coverslips are allowed to dry and then mounted with mowiol. All experiments are imaged with Leica DM6000 microscope; black and white images are taken with CCD camera and later processed with ImageJ or photoshop. Images were quanitified using ImageJ. With the exception of Mis12 and Ndc80 in figure R21 were Definiens was used to measure total fluorescence per nuclei. To measure the distance between centromeres ImageJ was used.

3.6 Immunoprecipitation

For immunoprecipitation of condensin II from the extract 5μg of antibody is added to 55μl of high speed CSF extract, and incubated for 2 hours on ice. 10μl of PureProteome protein A magnetic beads are then added and incubated at 4°C with rotation for 1 hour. Beads are washed five times with XBE2 buffer then resuspended in 10μl of 1XSDS sample buffer. 5μl is run on western blot alongside 1μl of the unbound fraction.

3.7 RNA interference in human cell lines

The siRNA duplexes used to repress CENP-C and CENP-T are dharmacon smartpools CENP-C1 (SMARTpool siGENOME CENP-C, NM-003251-03) and CENP-T (SMARTpool siGENOME CENP-T M-014577-02). Exponentially growing HeLa cells are seeded in wells with or without poly-L-lysine-coated coverslips and transfected with 100 nM oligo RNA duplexes using Dharmafect 24h and 48h after seeding. After another 22h 100ng/ml nocodazole is added and incubation proceeds for 2 hours before beginning immunofluorescence protocol.

For depletion of condensin II a single siRNA duplex against hCAP-D3 is used (sense sequence: CAUGGAUCUAUGGAGAGUAUU). Exponentially growing 293T-REX-GFP-CENP-A cells previously generated in the lab are seeded and transfected with 100 nM oligo RNA duplexes using Dharmafect 24h and 48h after seeding. 24 hours after the second transfection cells are treated for 1 hour with doxycycline to induce expression of GFP-CENP-A. Cells are then re-seeded on to poly-L-lysine-coated coverslips and left to grow for a further 24 hours before fixation.

4. Results

4. Results

4.1 Cell cycle recruitment of CCAN complexes

The Xenopus egg extract system has been used for a long time to study kinetochore assembly. Many of these studies have been carried out in non-cycled CSF extracts. There is increasing evidence that the centromeres and kinetochores on these uncycled chromosomes are different to those on cycled replicated chromosomes. We wanted to characterise the differences in these centromeres, as it can be important when contemplating previously published data and designing future experiments. The CCAN is made up of at least sixteen centromere proteins that have been divided into five functional subcomplexes. Four of the five subcomplexes (CENP-C, CENP-T-W-S-X, CENP-L-N, CENPH-I-K-M) have been described as having importance in the recruitment of at least one of the other five and the CENP-O-P-Q-U-R is downstream of all the others. In the *Xenopus laevis* cell free egg extract system we assemble '*de novo*' centromeres on a naïve chromatin template only containing CENP-A. We wanted to characterise when each of the four main subcomplexes binds to chromatin and builds the centromere.

We used antibodies for one member of each subcomplex, CENP-C and CENP-T antibodies that had already been raised and characterised in the lab plus we raised new antibodies against CENP-N and CENP-K (See materials and methods for antibody descriptions). We added demembranated sperm to CSF arrested extracts or interphase extracts and then cycled the extract into mitosis by adding cyclin B. Nuclei and chromosomes were fixed and then isolated by centrifugation through a glycerol cushion onto coverslips, they were then stained with the aforementioned antibodies. By immunofluorescence we saw CENP-C was present at centromeres in CSF, interphase and cycled mitotic centromeres (Figure R1a). CENP-T was not present on CSF centromeres but bound during interphase and remained present on mitotic centromeres (Figure R1b).

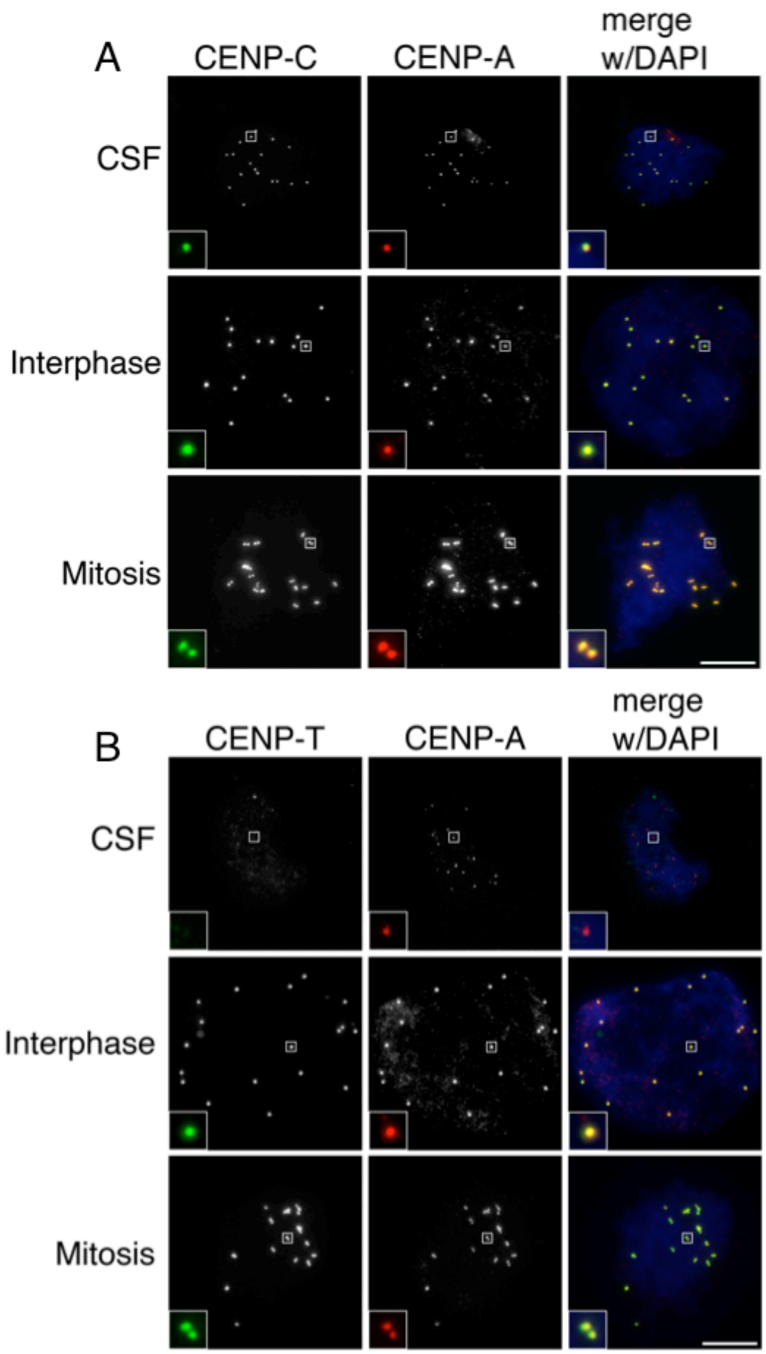


Figure R1. CENP-C is present on CSF centromeres while CENP-T binds in interphase. Localisation of CENP-C (A) and CENP-T (B) on CSF, interphase and cycled mitotic chromosomes. Sperm chromatin was added to either CSF extract or interphase extract then cycled to mitosis with cyclin B. Chromatin was fixed and stained with the indicated antibodies. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

By immunofluorescence we detected CENP-K at centromeres in interphase and cycled mitotic nuclei but not CSF nuclei (Figure R2). The CENP-N antibody could not clearly detect CENP-N at centromeres in CSF. There are some foci that appear to co-localise with some centromeres but they appear very similar to the non-specific staining (Figure R3a). CENP-N has proven difficult to study, as the full length recombinant protein is not stable without co-expression of CENP-L (Hinshaw and Harrison, 2013). We were able to express *Xenopus laevis* myc-CENP-N without co-expressing CENP-L using an in vitro reticulocyte system. When we added myc-CENP-N to the extract we could not detect myc at CSF centromeres (Figure R3b). In cycled mitosis both the antibody and myc-CENP-N give a clear centromeric signal. In interphase we did not detect CENP-N well at centromeres by immunofluorescence with the antibody, however when we added Myc-CENP-N to extracts this could be detected at interphase centromeres (Figure R3).

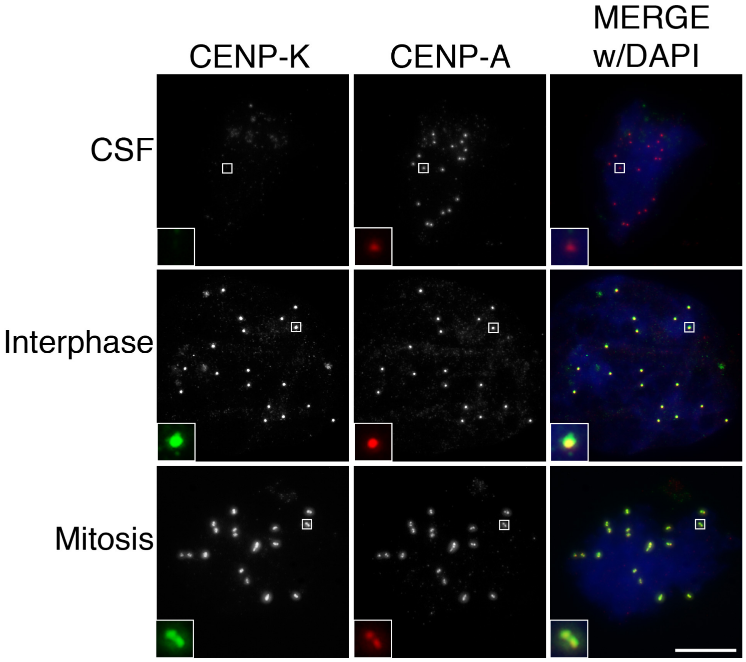


Figure R2. CENP-K is present on interphase and cycled mitotic centromeres. Localisation of CENP-K on CSF, interphase and cycled mitotic chromosomes. Sperm chromatin was added to either CSF extract or interphase extract then cycled to mitosis with cyclin B. Chromatin was fixed and stained with CENP-K antibody for immunofluorescence. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

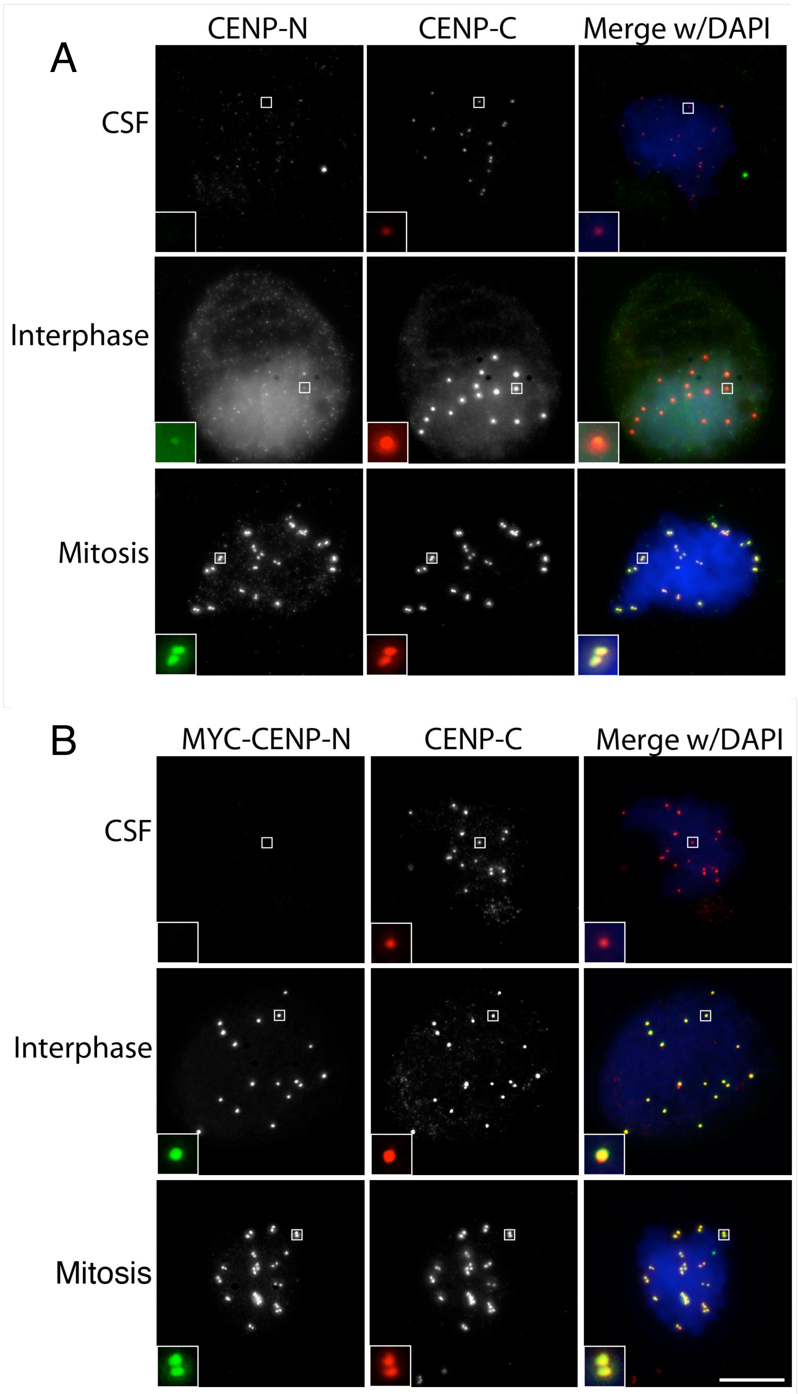


Figure R3. CENP-N can bind to interphase and cycled mitotic centromeres. Localisation of CENP-N (A) and myc-CENP-N (B) on CSF, interphase and cycled mitotic chromosomes. (**A)** Sperm chromatin was added to either CSF extract or interphase extract then cycled to mitosis with cyclin B. Chromatin was fixed and stained with the CENP-N antibody for immunofluorescence. Centromeres are marked with CENP-A. DNA was stained with DAPI. **(B)** As in (A) but myc-CENP-N translated in rabbit leukocytes was added to the extracts prior to sperm addition. Stained with anti myc antibody. Scale bar: 10μM.

We also performed an immunoblot of chromatin assembled in CSF, interphase and cycled mitotic extracts (Figure R4). The blot confirmed the results we saw by immunofluorescence for CENP-C and CENP-K. In the CENP-T we saw a band on the CSF chromatin but this was much lower than what is seen on interphase and cycled mitotic chromatin. This may be due to non-specific binding of the antibody that is also seen by immunofluorescence in CSF (Figure R1b). We could also detect CENP-N on chromatin in interphase however we could not detect it on mitotic chromatin. This could be explained by the antibody detecting the all over chromatin binding we see by immunofluorescence in interphase, the CENP-N bound to centromeres in mitosis may be below the antibody's limit of detection for immunoblot. Our results suggest that on CSF centromeres the only CCAN member present is CENP-C and the other complexes bind during interphase (Figure R5).

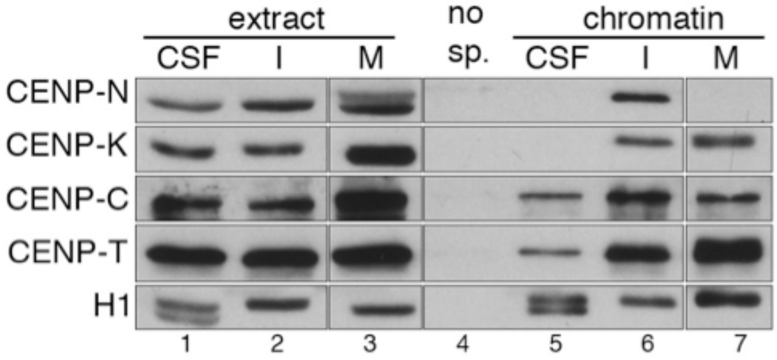


Figure R4. Chromatin binding of CENP-N, CENP-K, CENP-C and CENP-T in CSF, interphase and mitosis. Immunoblot of chromosomes assembled in CSF, interphase (I) or cycled mitotic (M) extracts. no sp. = control processed in the same way as other samples but with no sperm added. H1, loading control.

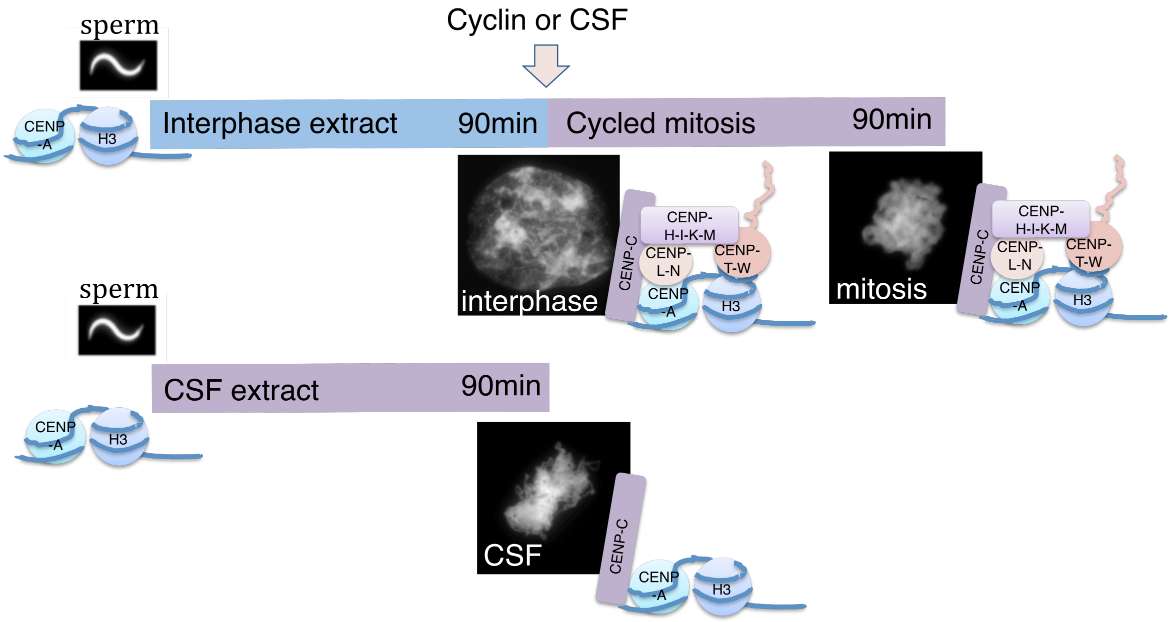


Figure R5. Sperm must cycle though interphase for the full CCAN to bind to centromeres. Summary of when CENP-C, CENP-T, CENP-N and CENP-K bind to centromeres in the Xenopus egg extract system.

4.2 Hierarchy of interactions in CCAN assembly

There is some discrepancy in the literature about the hierarchy of assembly of the CCAN subcomplexes and which ones are interdependent. There are differences between model systems, techniques and cell cycle stages. We decided to define this hierarchy in the Xenopus egg extract cell-free system. As the CSF centromeres only have CENP-C present we cannot use them to assess the hierarchy of assembly. Here we defined the interdependencies at interphase and cycled mitotic centromeres. Using antibodies crosslinked to magnetic beads we depleted each CENP individually from the soluble extract. Then we assembled chromosomes in the extract and looked how this affected the localisation of the other three CENPs at the centromere by immunofluorescence. Although CENP-K and CENP-N depletions are very good, less than 10% remains (Figure R6), we still detected a very small amount by immunofluorescence at centromeres in chromosomes assembled in depleted extracts (Figure R9 & R11). In the CENP-C and CENP-T depletions we did not detect any at centromeres (Figure R7 & R8). None of the CENPs we looked at caused co-depletion of each other when depleted from the extract (Figure R6). This suggested that they are part of separate subcomplexes.

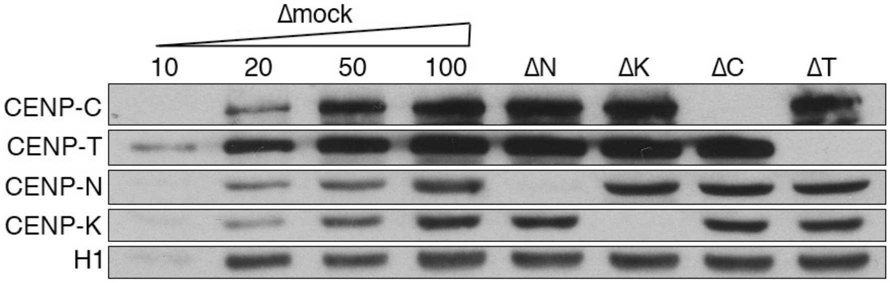
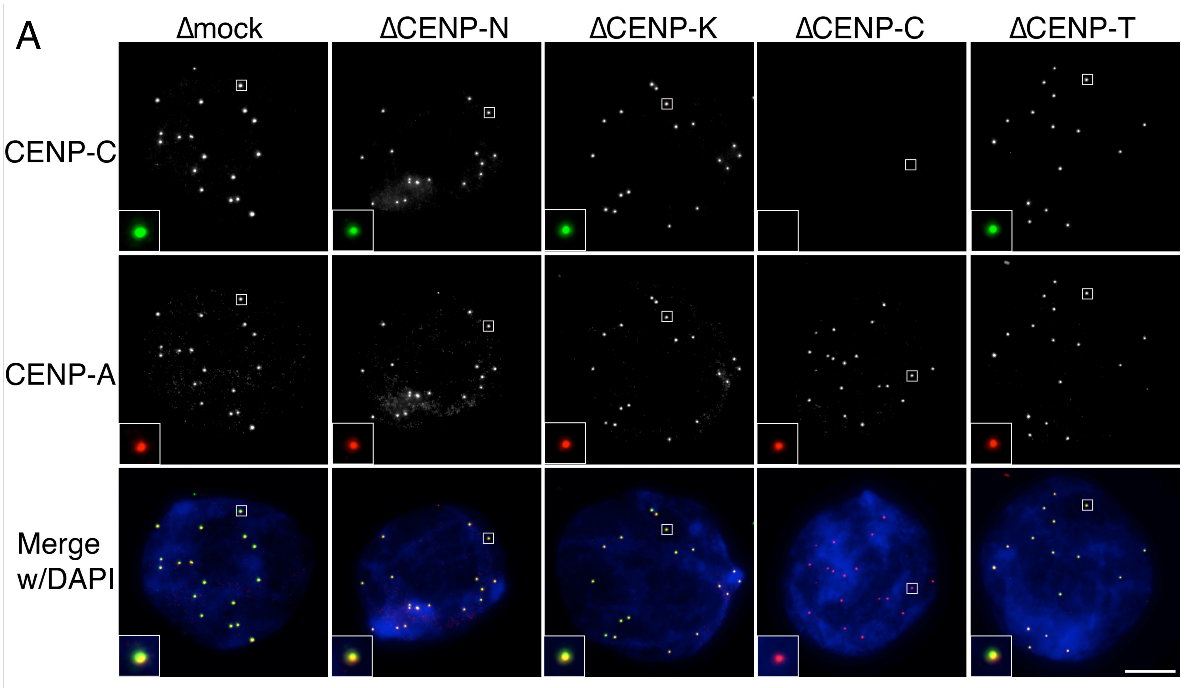


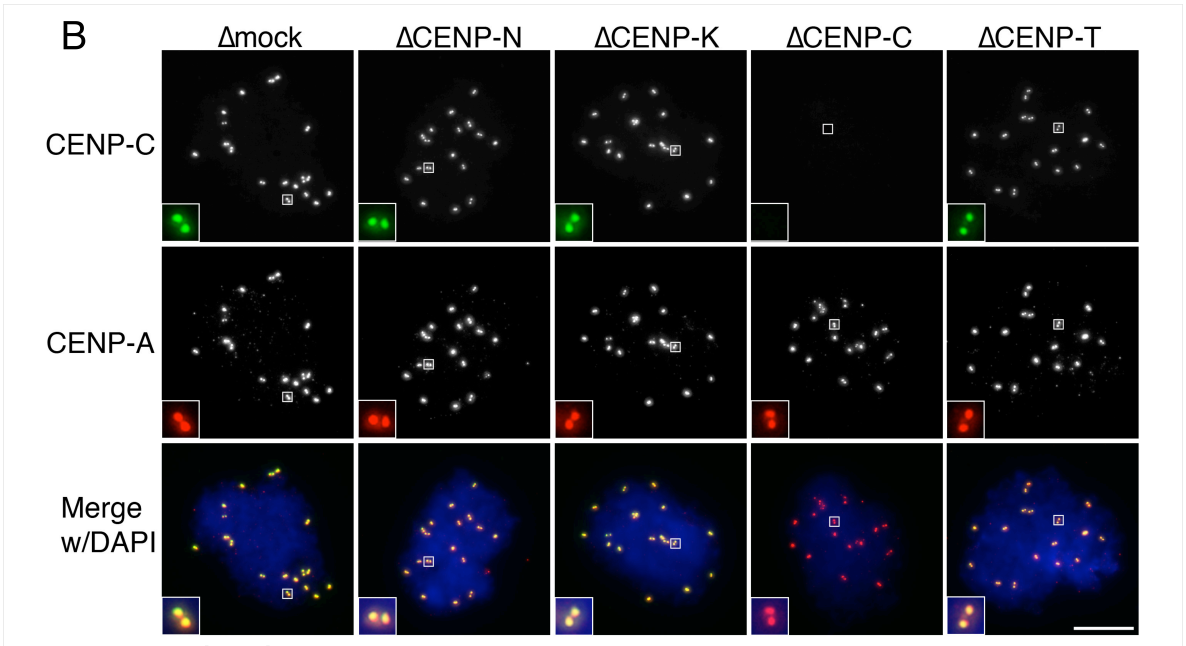
Figure R6. None of the subcomplexes co-deplete each other following immunodepletion. Immunoblot to show efficiency of depletion of CENP-C, CENP-T, CENP-N and CENP-K from soluble extract. Increasing amounts of mock-depleted extract, expressed as percentage, and aliquots of extracts depleted with specific antibodies, as indicated, were analyzed side by side to estimate the extent of each depletion. H1, loading control.

CENP-C is not obviously decreased at centromeres by the depletion of any of the other three subcomplexes (CENP-T, CENP-N, CENP-K) in mitosis (Figure R7b). This is in agreement with what has been seen in many other systems. In interphase there may be a small reduction of CENP-C with depletion of the other complexes (Figure R7a). It is clear though that CENP-C is able to bind to centromeres in the absence of each of the other complexes. This is consistent with its reported direct interaction with CENP-A.

CENP-T was completely removed from mitotic centromeres in CENP-K depleted extracts but a substantial amount remained at interphase centromeres. Suggesting that CENP-T is completely dependent on CENP-K in mitosis but only partially in interphase. In the CENP-N depletion we see a large reduction in both interphase and mitosis but not complete loss. With CENP-C depletion we see a reduction of CENP-T but not loss in both interphase (Figure R8a) and mitosis (Figure R7a). Thus CENP-T has a degree of dependency on all the other three complexes but a population, at least, is able to bind to centromeres independently. It would be interesting to perform double depletions to see if the independency is due to different populations being dependent on different complexes. For example, co-deplete CENP-K and CENP-C and see if CENP-T is lost at interphase centromeres.



interphase



mitosis

Figure R7. CENP-C centromeric localisation is not dependent on CENP-N, CENP-K or CENP-T. (A) Interphase extract was depleted of CENP-N, CENP-K, CENP-C or CENP-T and sperm chromatin added. Nuclei were fixed after 90 mins and stained for CENP-C. **(B)** As in (A) but cyclin was added after the first 90 mins, to cycle the extract in to mitosis, and they were left for a further 90 mins before fixing. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

Immunofluorescence shows that in the absence of CENP-C in interphase CENP-K is reduced but present at centromeres whereas in mitosis it is much more reduced. Depletion of CENP-N causes complete loss of CENP-K in interphase and almost complete loss in mitosis. CENP-T depletion may cause a slight reduction in CENP-K but it is clearly able to bind to centromeres in CENP-T's absence (Figure R9).

Defining what happens to CENP-N was more complicated. As in interphase by immunofluorescence the antibody did not appear to recognise CENP-N well at centromeres (in some experiments we saw a weak centromeric signal). By immunoblot CENP-N on chromatin did not seem to be affected by the depletion of the other subcomplexes in interphase (Figure R12a). When looking at the immunofluorescence of the CENP-N antibody in interphase we saw a lot of non-specific binding (Figure R3a), if this also occurred with the purified chromatin for immunoblot it could explain why we are unable to detect a difference. However if we added myc-CENP-N to the extract and looked at it by immunofluoresence we could see depletion of either CENP-K or CENP-C prevented myc-CENP-N from accumulating at centromeres. We would like to further characterise the CENP-L-N complex by raising an antibody against CENP-L. We expressed Myc-CENP-L but it didn't localise to centromeres in CSF, interphase or mitosis. This is likely because it was not able to incorporate into the endogenous complex.

Immunoblot of total chromatin is a less accurate way to look at recruitment of centromere proteins than immunofluorescence. Discrepancies can occur when looking at the whole chromatin fraction because of non-specific binding to non-centromeric chromatin. It is also possible that, since chromatin is not fixed before centrifugation through a sucrose cushion, looser interactions may be lost. Nonetheless this is a useful supporting method. We isolated chromatin assembled in both interphase and mitotic extracts depleted of each CCAN member and the immunoblots could support some of our observations. Depletion of CENP-T does not cause reduction of the other complexes. CENP-T is reduced more in the CENP-N and CENP-K depletion that in CENP-C depletion. CENP-K is reduced in CENP-N and CENP-C depletions (Figure R12).

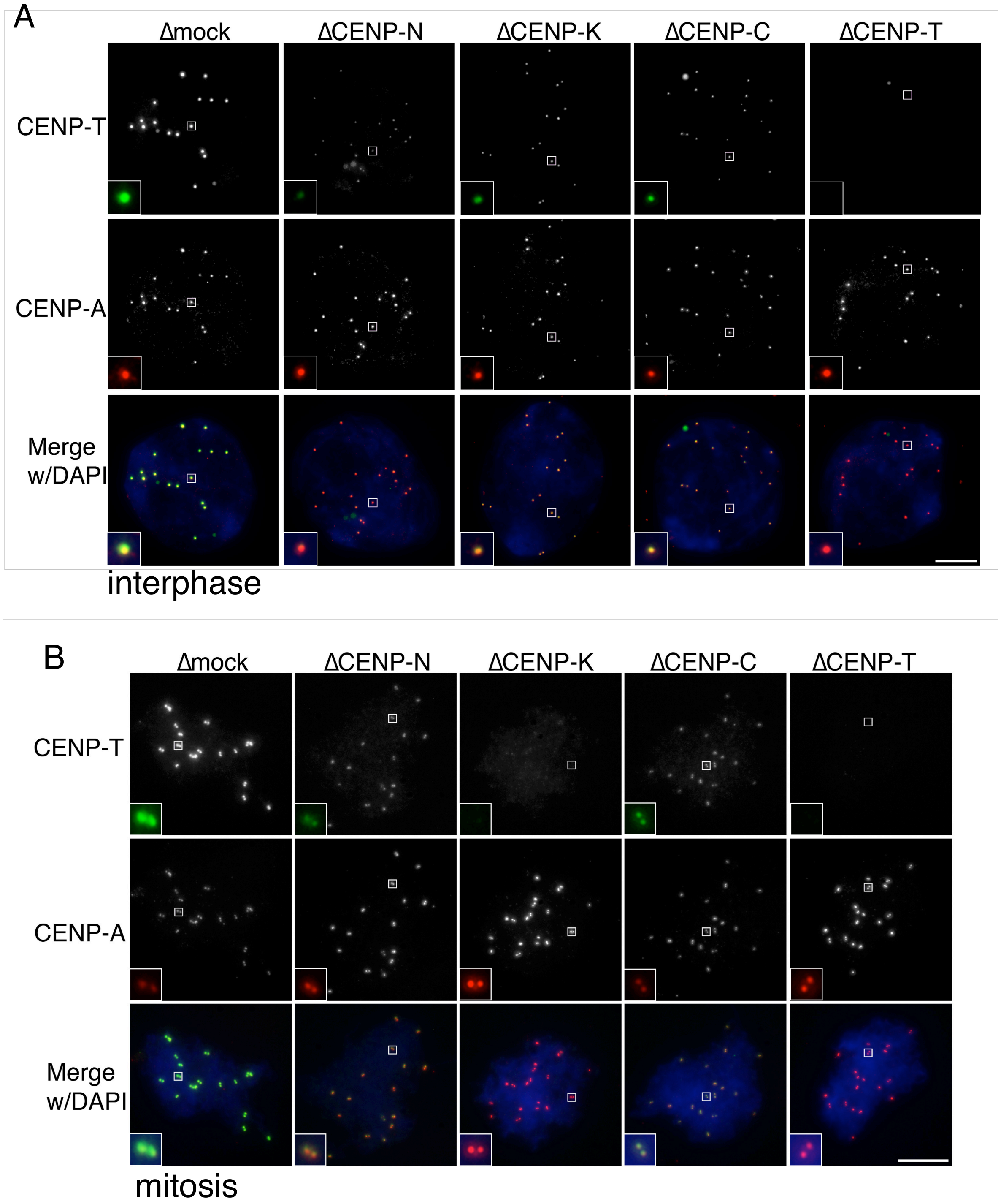


Figure R8. CENP-T has some dependency on CENP-N, CENP-K and CENP-C but is able to bind to centromeres in the absence of CENP-C. (A) Interphase extract was depleted of CENP-N, CENP-K, CENP-C or CENP-T and sperm chromatin added. Nuclei were fixed after 90 mins and stained for CENP-T. **(B)** As in (A) but cyclin B was added after the first 90 mins, to cycle the extract in to mitosis, and they were left for a further 90 mins before fixing. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

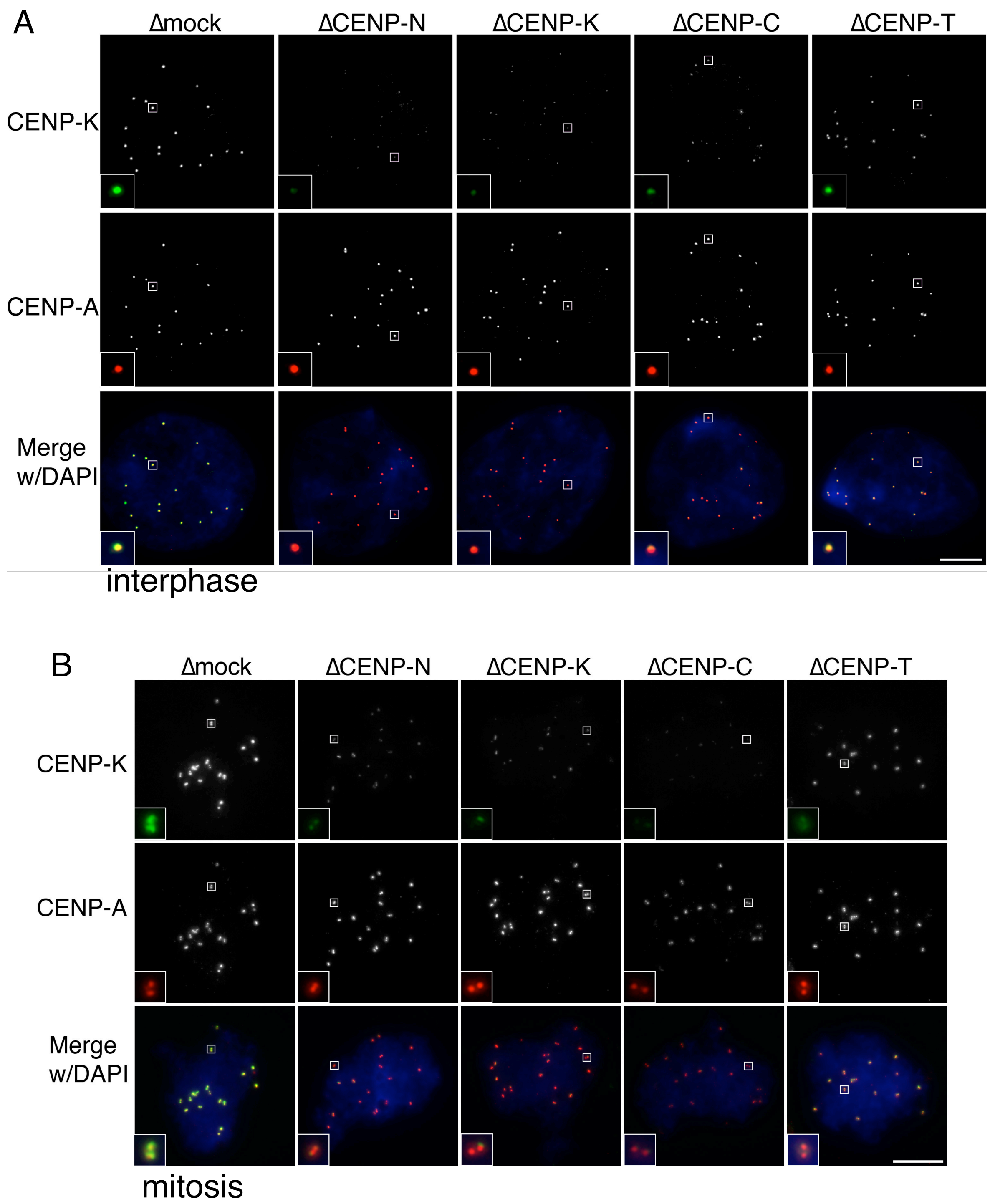


Figure R9. CENP-K is dependent on CENP-N but only partially dependent on CENP-C. (A) Interphase extract was depleted of CENP-N, CENP-K, CENP-C or CENP-T and sperm chromatin added. Nuclei were fixed after 90 mins and stained for CENP-K. **(B)** As in (A) but cyclin B was added after the first 90 mins, to cycle the extract in to mitosis, and they were left for a further 90 mins before fixing. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

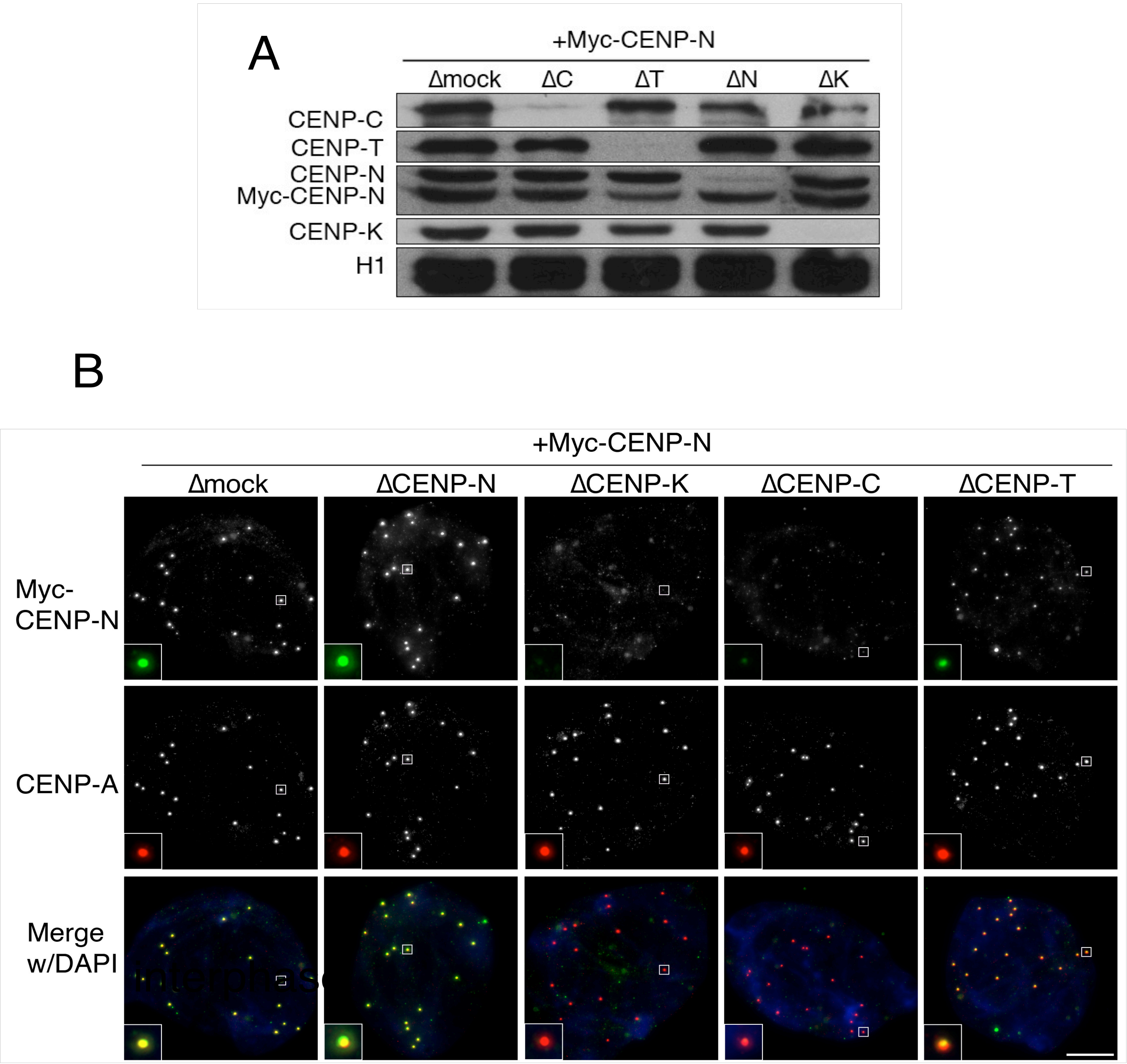


Figure R10. myc-CENP-N requires CENP-K and CENP-C to target to interphase centromeres. **(A)** Immunoblot to show myc-CENP-N, translated by TNT system in rabbit leukocytes, was added to interphase extract depleted of CENP-N, CENP-K, CENP-C or CENP-T. H1, loading control. **(B)** Sperm chromatin was added to the extracts, nuclei were fixed after 90 mins and stained with a myc recognising antibody. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

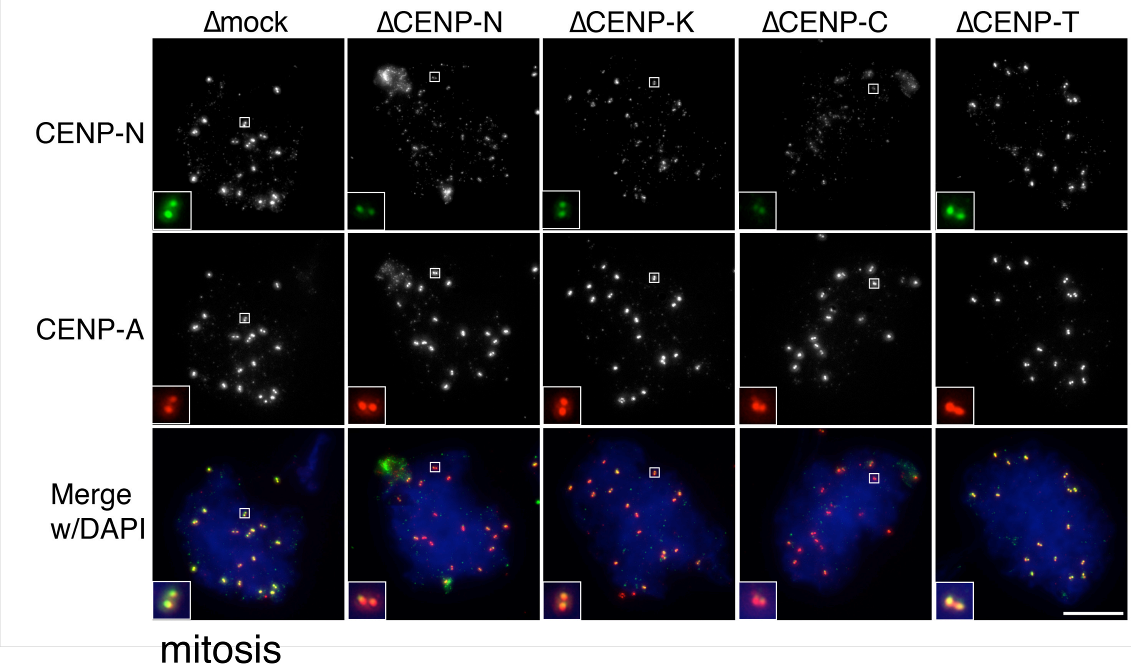


Figure R11. CENP-N recruitment to mitotic centromeres is reduced in the absence of CENP-C or CENP-K. Interphase extract was depleted of CENP-N, CENP-K, CENP-C or CENP-T and sperm chromatin added, after 90 mins cyclin B was added, to cycle the extract in to mitosis, and they were left for a further 90 mins before fixing. Chromosomes were stained for CENP-N. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10*μ*M.

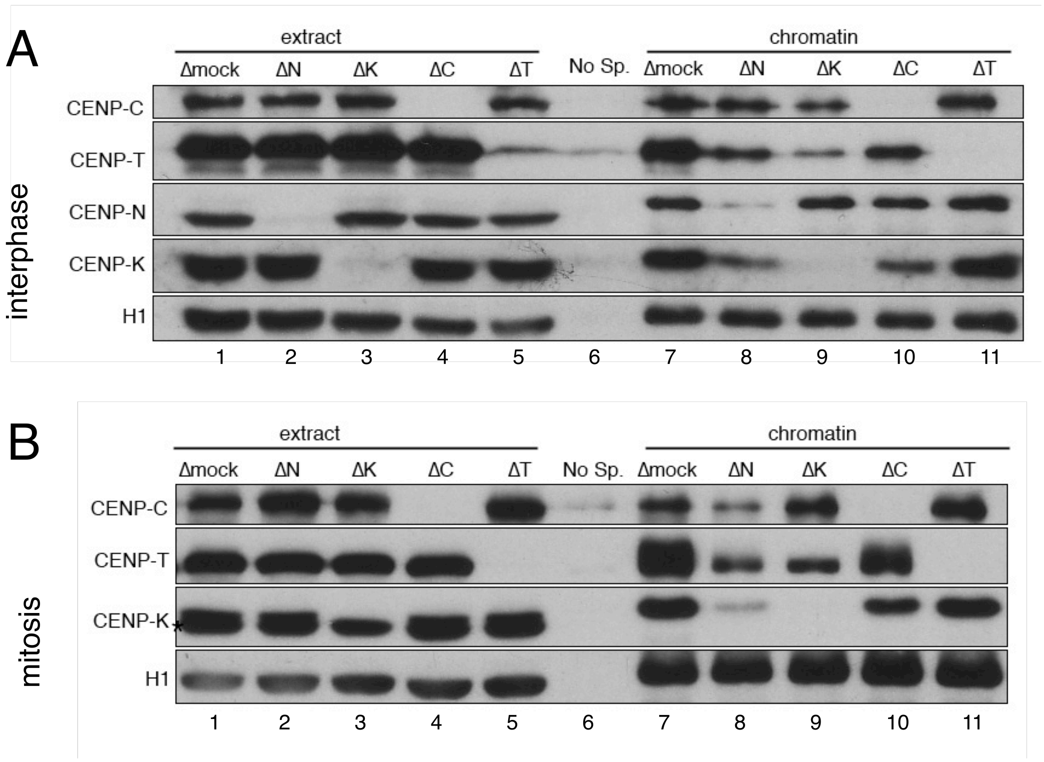


Figure R12. Total chromatin bound centromere proteins in absence of other CCAN members. (A) Interphase extract was depleted of CENP-N, CENP-K, CENP-C or CENP-T and sperm chromatin added. After 90 mins chromatin was isolated from the extract by spinning through a sucrose cushion (see materials & methods). **(B)** As in (A) but cyclin B was added after the first 90 mins, to cycle the extract in to mitosis, and they were left for a further 90 mins before chromatin isolation. * indicates non-specific band present when blot mitotic extract cycled by adding cyclin B. H1, loading control.

In summary our most important observations were that CENP-C binding is not dependent on any of the other subcomplexes. CENP-T is not required for the binding of any of the other sub-complexes. CENP-N and CENP-K are interdependent. CENP-T does not require CENP-C to bind to centromeres but to keep the normal levels CENP-C is required. CENP-T is more dependent on CENP-N and CENP-K than CENP-C. We did not see much of a cell cycle dependent difference apart from CENP-T's dependency on CENP-K being much higher in mitosis than interphase (Table RT1).

Interphase

depleted protein	effect			
	CENP-C	**CENP-T**	**myc-CENP-N**	**CENP-K**
control	++++	++++	+++	++++
CENP-C	-	++	+	++
CENP-T	+++	-	+++	+++
CENP-N	+++	+	++++	+
CENP-K	+++	++	-	+

Mitosis

depleted protein	effect			
	CENP-C	**CENP-T**	**CENP-N**	**CENP-K**
control	++++	++++	++++	++++
CENP-C	-	++	+	-
CENP-T	++++	-	++++	++
CENP-N	++++	++	+	+
CENP-K	++++	-	+	+

Table RT1. Summary of CCAN interdependencies. Qualitative assessment of levels of each CENP left after the depletion of the others.

One contradicting result is that CENP-T requires CENP-K at mitotic centromeres but in the CENP-C depletion where we see very little CENP-K by immunofluorescence we still see a substantial amount of CENP-T. When we looked by immunoblot in the CENP-C depletion we could still see a substantial amount of CENP-K on chromatin. One possibility is that the CENP-T antibody is more sensitive than the CENP-N and CENP-K antibodies for immunofluorescence. We would like to further expand on these CCAN characterisation experiments. Along with the CENP-L antibody we would also like to raise an antibody against CENP-S, this is in principle a part of the CENP-T-W-S-X complex. There are some reports that CENP-S loads after CENP-T. It would be interesting to see if it behaves the same as CENP-T in this system. One possibility is that there are two populations of CENP-T that have different requirements for loading and maintenance and different functions. This is supported by the fact we have seen that CENP-W is more greatly loaded in mitosis than in interphase (Krizaic et al, 2015).

4.3 Interplay of centromere and kinetochore assembly with Sgo1 recruitment

The next objective was to ascertain which aspects of centromere and kinetochore assembly were required for Sgo1 recruitment. Sgo1 specifically localises to centromeres during mitosis. The main known requirement for Sgo1 recruitment to the centromere region is the phosphorylation of histone H2A by the kinase Bub1. Bub1 accumulation at kinetochores requires phosphorylation of KNL1 by the kinase Mps1. KNL1 is recruited to kinetochores through an interaction with the Mis12 complex. We first characterised the presence of known proteins important for Sgo1 recruitment at CSF and cycled mitotic chromosomes. All the proteins were present at both types of centromere although the signals were much stronger on cycled mitotic chromosomes (Figure R13 & R14).

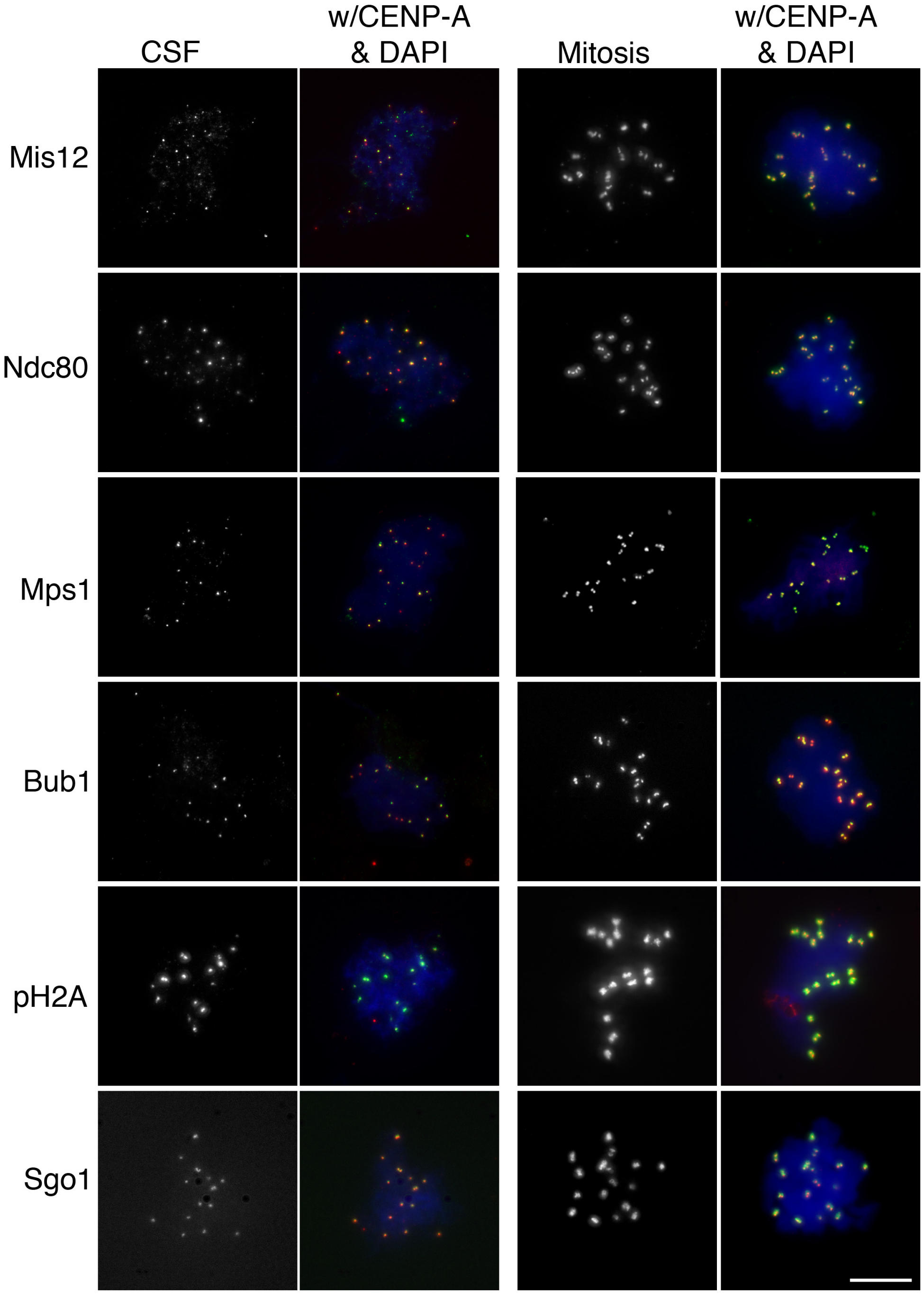


Figure R13. Mis12, Ndc80, Mps1, Bub1, pH2A and Sgo1 can be seen at CSF and cycled mitotic centromere. Sperm was added to either CSF extracts and left for 90 mins or to interphase extracts for 90 mins before adding cyclin for a further 90 mins. Chromosomes were fixed and stained with the indicated antibodies. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

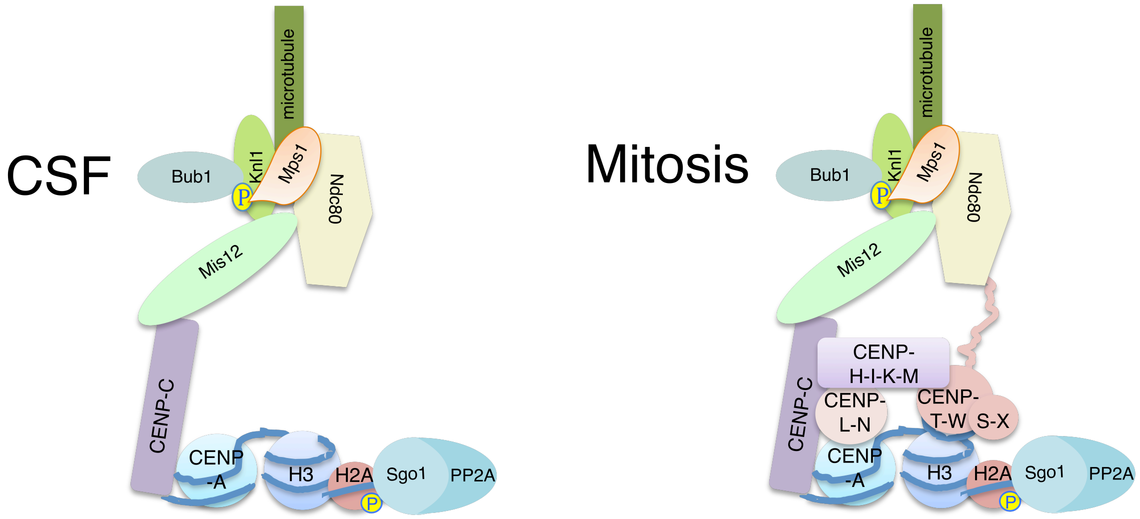


Figure R14. Sgo1 and proteins required for its recruitment are present at both CSF and cycled mitotic centromeres. Summary of relevant proteins present at CSF and cycled mitotic centromeres.

4.3.1 Role of the CCAN in Sgo1 recruitment

As a large number of centromere and kinetochore proteins have been described as having a dependency on CENP-C we decided to use it as a starting point for our study. We depleted CENP-C from the *Xenopus laevis* egg extract and assembled chromosomes in it. In CSF chromosomes depletion of CENP-C caused the complete loss of Sgo1 at centromeres (Figure R15a). However, in cycled mitotic chromosomes depletion of CENP-C only caused a reduction of Sgo1 (Figure R15b).

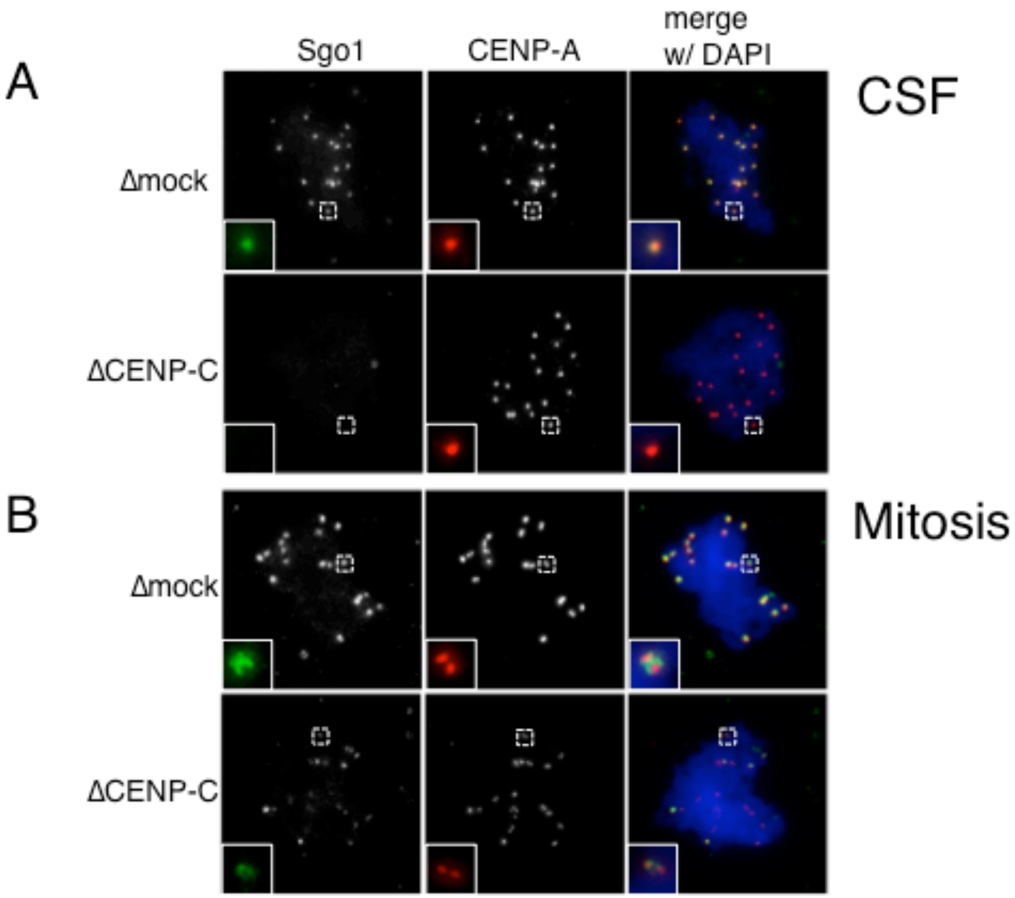


Figure R15. Sgo1 recruitment is completely dependent on CENP-C at CSF centromeres but not cycled mitotic centromere. (A) CSF extracts were depleted of CENP-C. Sperm was added and left for 90 mins, chromosomes were fixed and stained for Sgo1. **(B)** The depleted extract used in (A) was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Sgo1. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

This meant that in the replicated chromosomes there must be another protein capable of recruiting Sgo1 independently of CENP-C that is not present at CSF centromeres. It is known that cohesin only binds to chromatin during interphase, for this reason in the xenopus egg extract system cohesin is not present on CSF chromosomes (Losada et al., 1998). Therefore, we thought that cohesin might be the protein responsible for the remaining Sgo1. However, when we simultaneously depleted both CENP-C and cohesin we still did not completely remove Sgo1 (Figure R16).

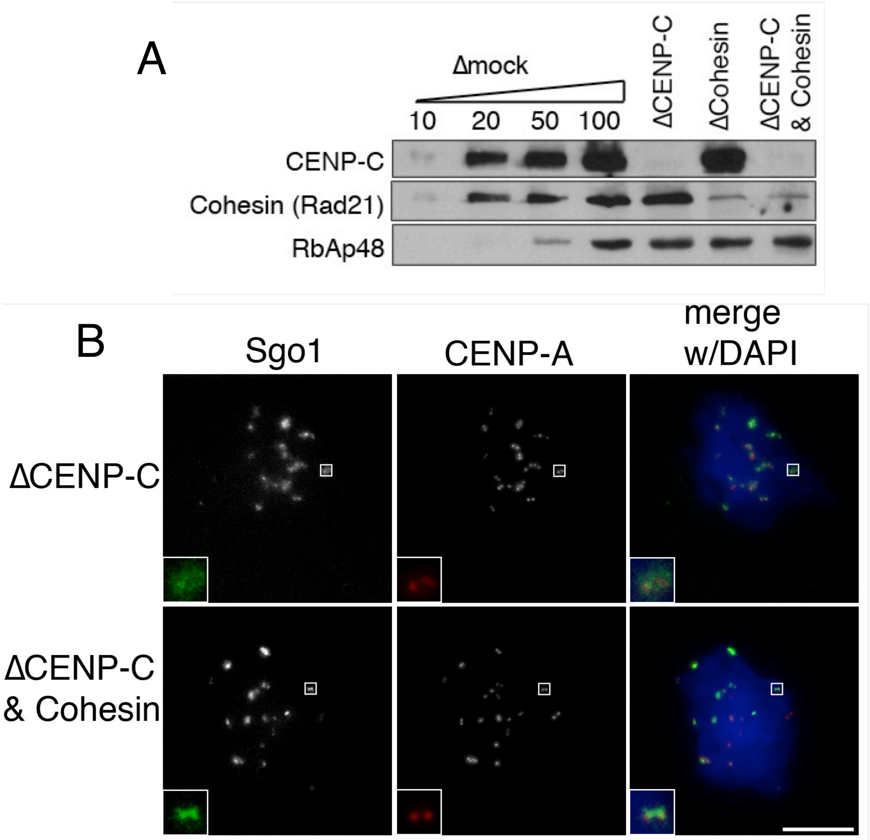


Figure R16. Sgo1 recruited independently of CENP-C is not dependent on cohesin. (A) Immunoblot of extract to show efficiency of depletion of CENP-C and cohesin. RbAp48, loading control. **(B)** The depleted extract was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Sgo1. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

As cohesin was not the protein responsible for the recruitment of Sgo1 in the absence of CENP-C we had to look for another candidate. As we showed earlier in the previous section CENP-T was not present in CSF but was in replicated mitotic chromosomes. Also, although reduced, a substantial amount of CENP-T remains at centromeres in the CENP-C depletion (Figure R8b, (Krizaic et al., 2015)). We saw that depletion of either CENP-C or CENP-T caused a reduction in Sgo1 at centromeres of chromosomes assembled in cycled extracts. Importantly a combined depletion of both completely removed Sgo1 from centromeres (Figure R17). This result suggested that both CENP-C and CENP-T could independently recruit Sgo1.

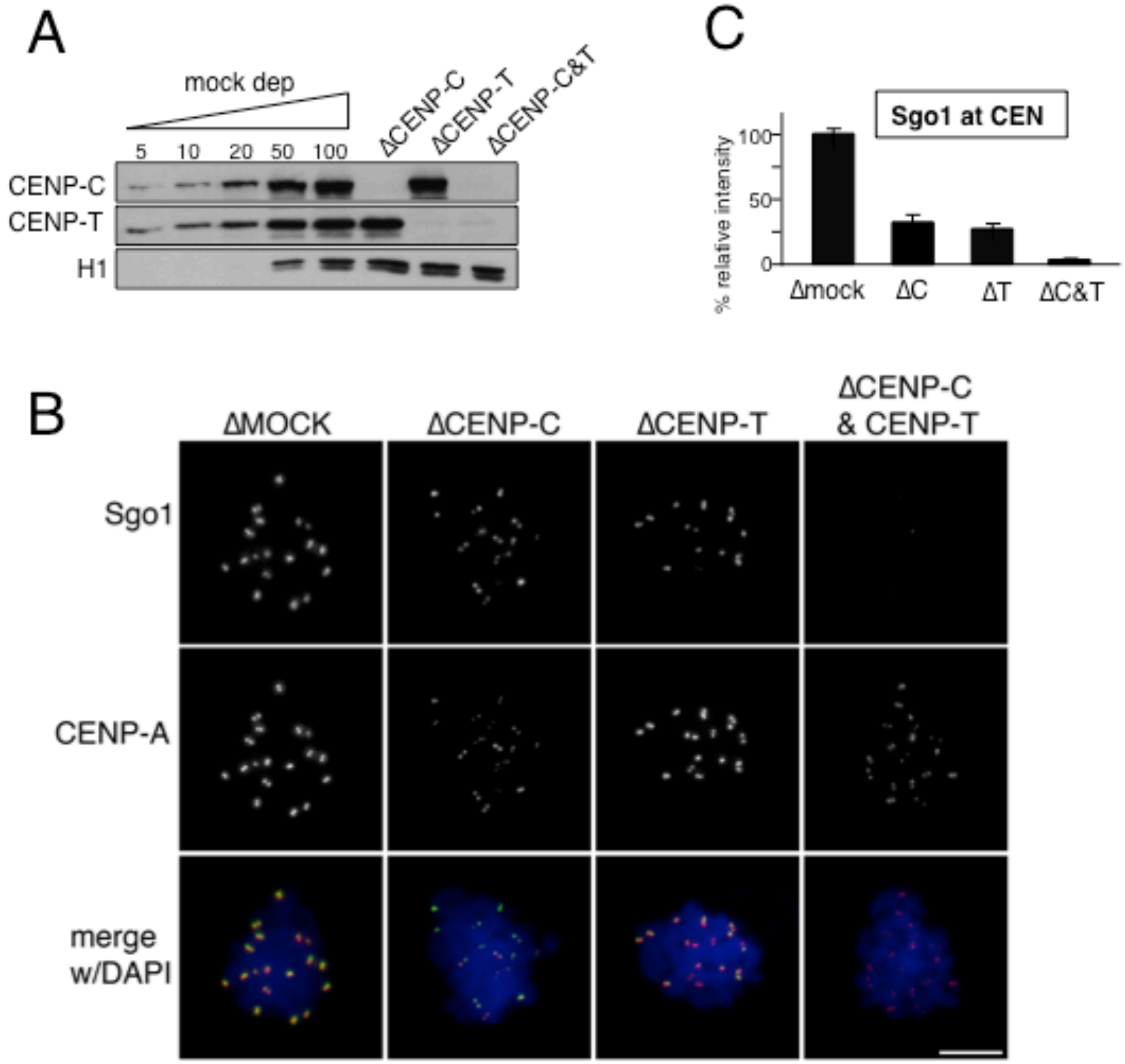


Figure R17. Sgo1 can be recruited by either CENP-C or CENP-T. (A) Immunoblot of extract to show efficiency of depletion of CENP-C, CENP-T or both combined. H1, loading control. **(B)** The depleted extract was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Sgo1. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM. **(C)** Quantification of (B) average fluorescence per centromere pair, compared to control. n= >30 nuclei from 3 separate experiments. Error bars, SEM.

It has been well established that one requirement for Sgo1 recruitment is phosphorylation of histone H2A at threonine 120 (pH2A) by the kinase Bub1. We therefore decided to establish the contribution of CENP-C and CENP-T to Bub1 centromere targeting and H2A phosphorylation. We generated a new antibody to recognise this phosphorylation in

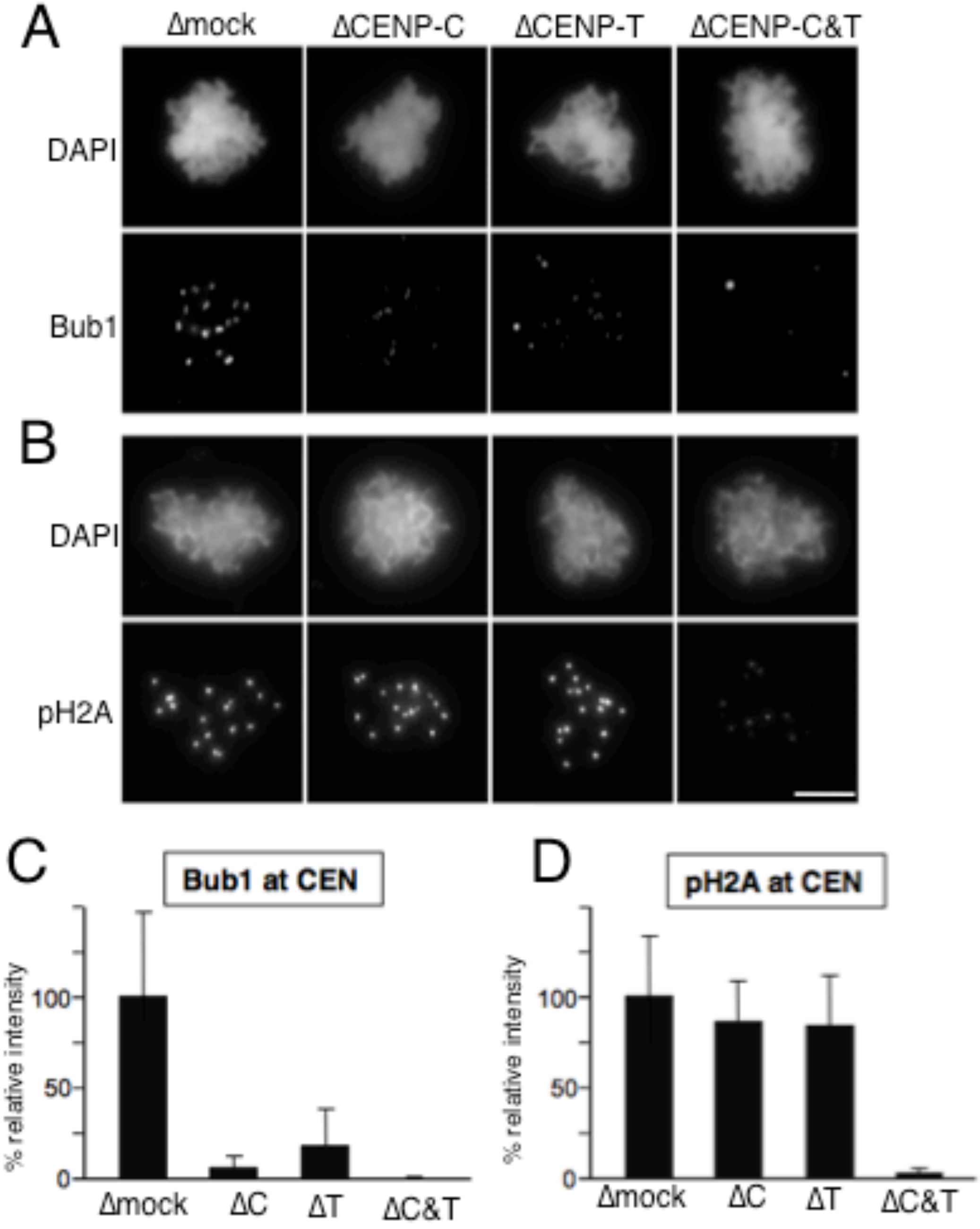


Figure R18. Bub1 can be recruited by either CENP-C or CENP-T. (A) Extract depleted of either CENP-C, CENP-T or both was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Bub1. Centromeres are marked with CENP-A. DNA was stained with DAPI. Quantification of average fluorescence per centromere pair, compared to control. n= >30 nuclei from 3 separate experiments. **(B)** as in (A) but chromosomes were stained for phosphoH2A T120. Scale bar: 10μM. Error bars, SEM.

Xenopus laevis (see materials and methods). We observed a great reduction in Bub1 staining at centromeres of chromosomes lacking CENP-C or CENP-T and complete removal in the double depletion (Figure 18a). These very low levels of Bub1 were nonetheless sufficient for a full or slightly reduced pH2A signal (Figure 18b).

Immunoblot analysis of chromatin assembled in cycled extracts lacking CENP-C, CENP-T or both corroborated the partial reduction of Sgo1 in the single depletions as well as the more dramatic effect of either CENP-C and CENP-T depletion (Figure R19 lane 5) or Bub1 depletion (Figure R19 lane 6).

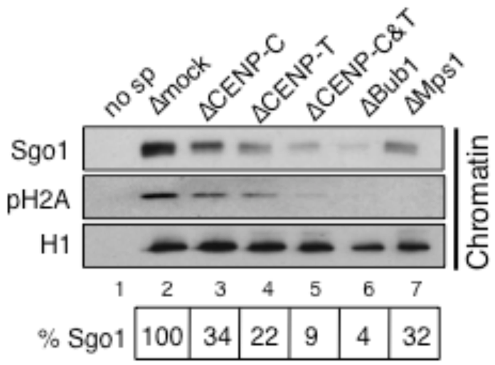


Figure R19. Sgo1 is reduced on chromatin in the absence of CENP-C or CENP-T. Immunoblot of chromatin assembled in extracts depleted of CENP-C (lane 3), CENP-T (lane 4), both CENP-C and CENP-T (lane 5), Bub1 (lane 6) or Mps1 (lane 7). A sample that was isolated in the same way but without sperm added (no sp.) serves as negative control (lane 1). H1 serves as a loading control; quantification of Sgo1 band is given relative to mock control and corrected for loading.

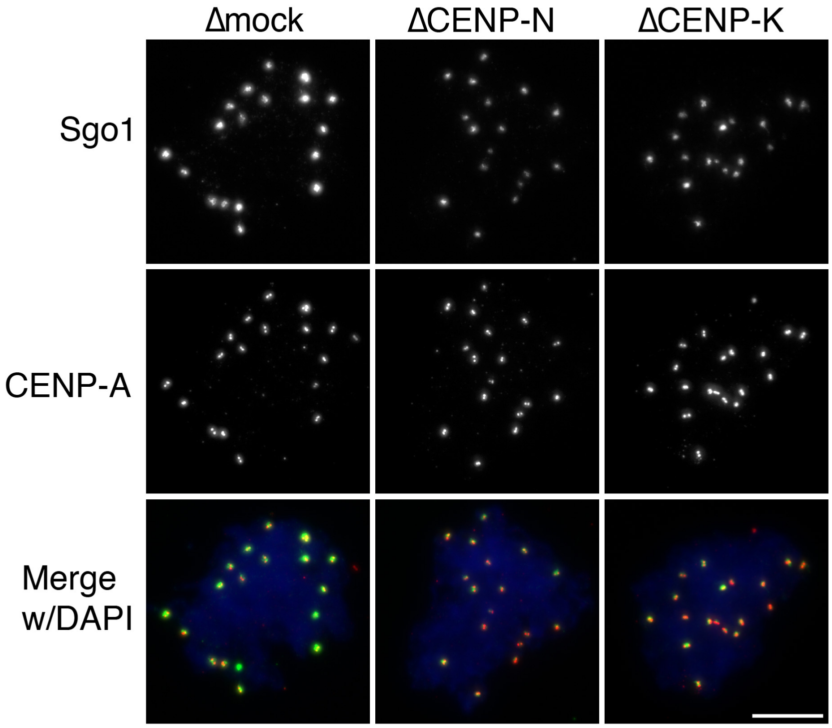
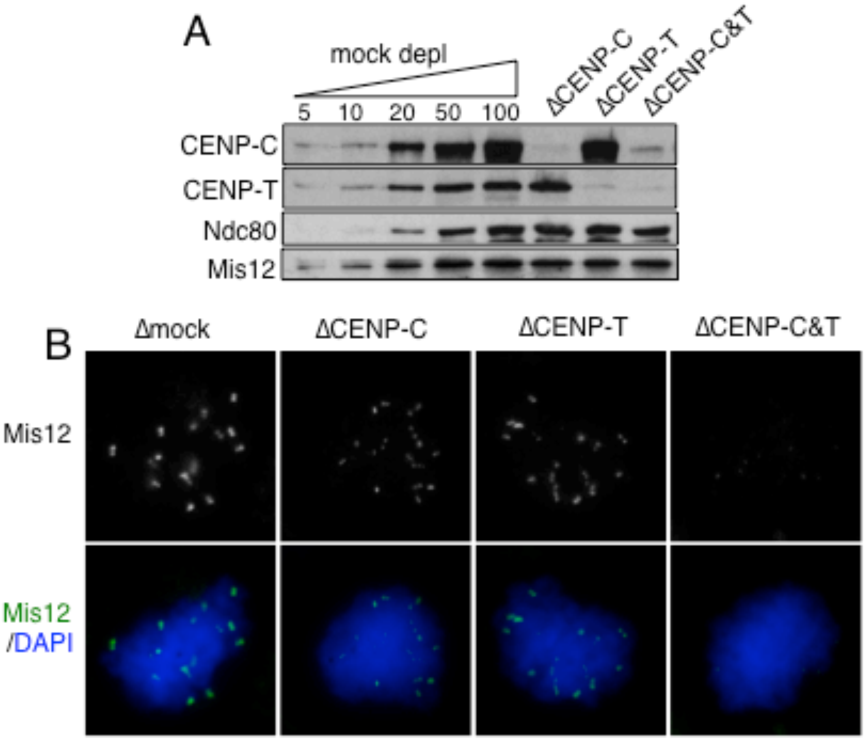


Figure R20. CENP-N and CENP-K are not required for Sgo1 recruitment. (A) cycled mitotic chromosomes assembled in extract that was depleted of CENP-N or CENP-K. Chromosomes were fixed and stained for Sgo1. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10*μ*M.

To assess whether CENP-N or CENP-K are required for Sgo1 recruitment we depleted them from the extract and assembled cycled mitotic chromosomes. There is a decrease in Sgo1 (Figure R20) consistent with the decrease in CENP-T (Figure R8b) we see in these conditions.

4.3.2 CENP-T recruitment of the KMN network

Both CENP-C and CENP-T play a role in recruiting outer kinetochore proteins. Bub1 interacts with KNL1, which is part of the KMN network along with Mis12 and Ndc80 complexes. We checked the effect of depleting CENP-C, CENP-T or both on Mis12 (Figure R21b) and Ndc80 (Figure R21c). Both Mis12 and Ndc80 were reduced at cycled mitotic centromeres while their levels in the extract remained unchanged (Figure 21a). The reduction in Bub1 could be attributed to the similar reduction in Mis12 (to which KNL1 binds) seen in both conditions. As Bub1 is a kinase a small amount is sufficient to produce a strong signal. If Sgo1 interacts directly with pH2A, as has been suggested (Liu et al., 2015), then why we see a reduction in Sgo1 is not clear and requires further investigation. It could be due to loss of other suggested recruitment pathways such as CENP-N (Hinshaw and Harrison, 2013) or HP1 (Yamagishi et al., 2008).



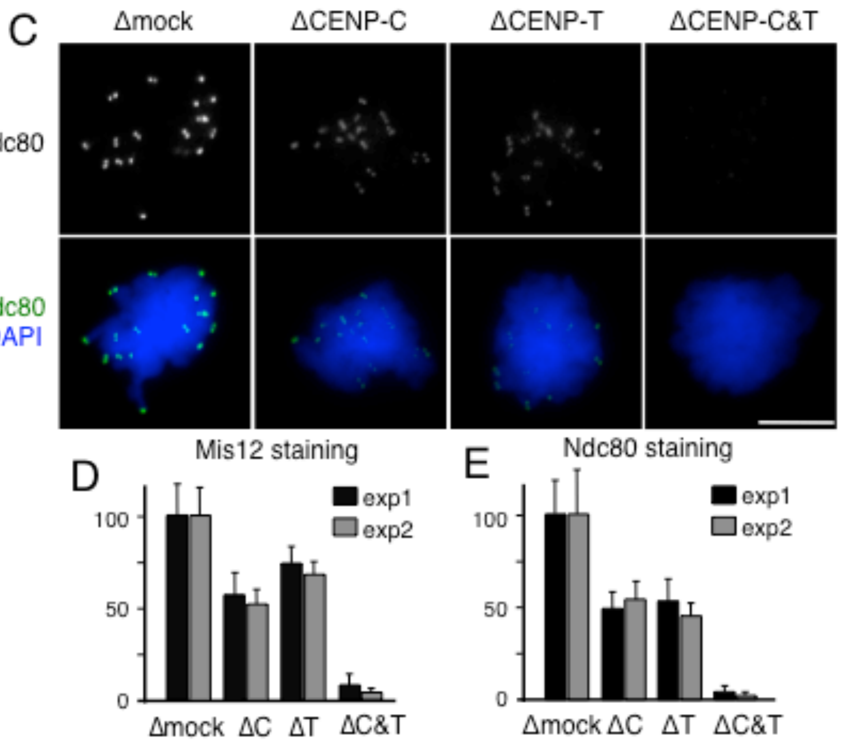


Figure R21. Mis12 and Ndc80 can be recruited by either CENP-C or CENP-T. (A) Immunoblot of extract to show efficiency of depletion and that Mis12 and Ndc80 are not co-depleted with CENP-C or CENP-T. **(B)** The depleted extract was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Mis12. Centromeres are marked with CENP-A. DNA was stained with DAPI. Quantification of total fluorescence per nuclei, compared to control. n= >10 nuclei per experiment. **(C)** As in (B) but chromosomes were stained for Ndc80. Scale bar: 10μM. Error bars, SEM.

4.3.3 Role of Mps1 in Sgo1 recruiment

Mps1 phosphorylates KNL1 creating the signal for Bub3 binding and therefore Bub1 accumulation at the kinetochore. This had been studied in the context of the spindle assembly checkpoint (SAC), but the necessity of Mps1 in Sgo1 recruitment had not been investigated. In the *Xenopus laevis* egg extract system, in the conditions we use, the SAC is not active. We saw that in Mps1 depleted extracts Bub1 was undetectable at mitotic centromeres (Figure 22).

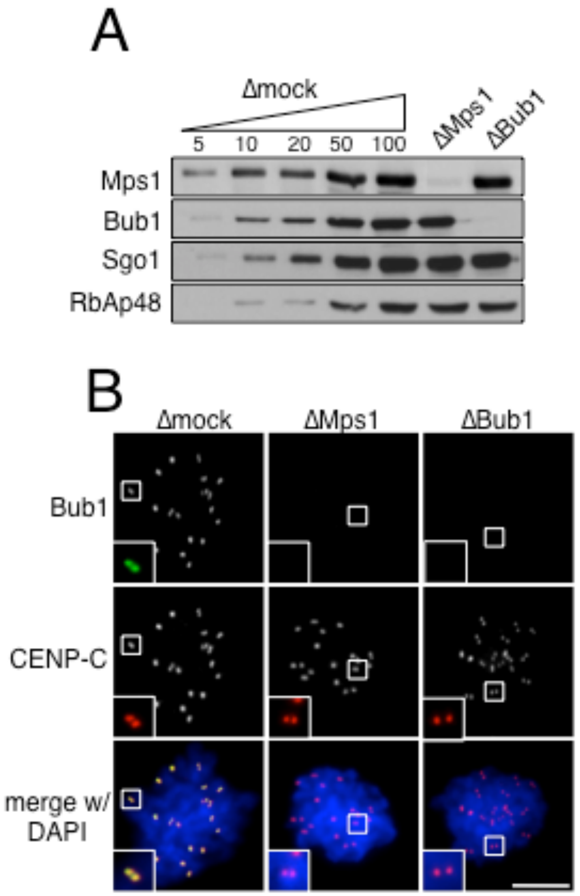


Figure R22. Bub1 recruitment to the centromere is lost in the absence of Mps1. (A) Immunoblot of extract to show efficiency of depletion of Mps1 and Bub1. RbAp48, loading control. **(B)** The depleted extract was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Bub1. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

Surprisingly when we depleted Mps1, unlike with Bub1 depletion, we still saw recruitment of a small amount of Sgo1 (Figure 23a).

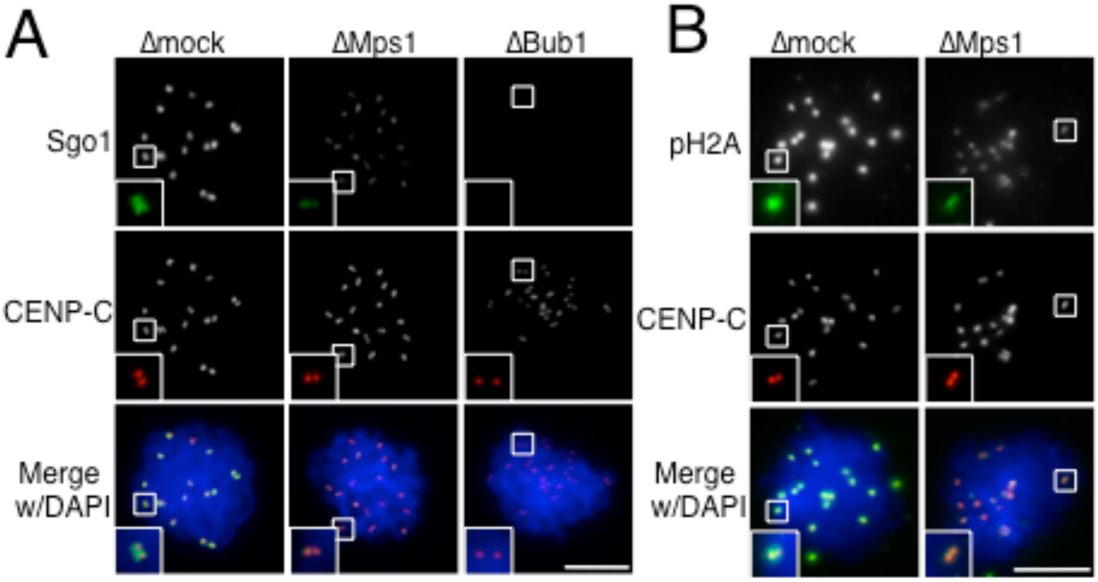


Figure R23. A small amount of Sgo1 can be recruited to centromeres in the absences of Mps1. (A) Extract depleted of Mps1 or Bub1 was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Sgo1. Centromeres are marked with CENP-C. DNA was stained with DAPI. Scale bar: 10μM. **(B)** As in (A) but chromosomes were stained for pH2A.

We also saw a reduced pH2A signal (Figure 23b). Therefore one possibility is that a small amount of Bub1 is still able to target to centromeres in the Mps1 depletion but we were not able to detect it. When we tried staining with a more sensitive Bub1 antibody we were able to see some signal remaining in the Mps1 depleton (Figure 24). This could be due to the depletion of Mps1 not being complete and only a small amount of Mps1 kinase being sufficient for phosphorylating KNL1 and recruiting Bub1. The other possibility is an Mps1 dependent recruitment of Bub1.

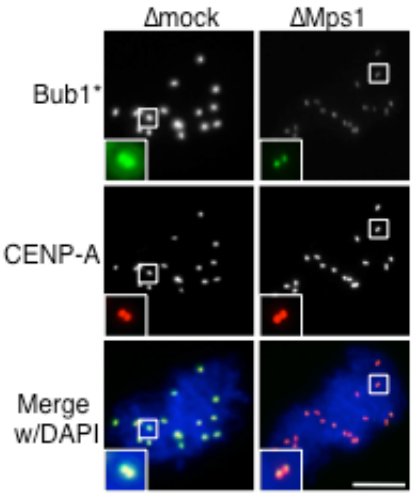


Figure R24. Some Bub1 can be recruited to centromeres in Mps1 depleted extracts. Interphase extract was depleted of Mps1 and sperm chromatin added, after 90 mins cyclin was added, to cycle the extract in to mitosis, and they were left for a further 90 mins before fixing. Chromosomes were stained with a different Bub1 antibody to the one used in Figure R22. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

We were interested to see if this pool of Sgo1 that didn't seem to require Mps1 was specifically dependent on either CENP-C or CENP-T. When we combined depletion of Mps1 with depletion of either CENP-C or CENP-T we similarly reduced the residual Sgo1 (Figure R25b). Disrupting the CCAN and therefore kinetochore affects the binding platform for Mps1 and could therefore prevent residual Mps1 functioning. However, it is also possible we are affecting an Mps1 independent pathway in this condition.

As another method to look at the importance of Mps1 in Sgo1 recruitment we decided to use a chemical inhibitor. Mitotic chromosomes were assembled and then thirty minutes before fixing 50μM reversine was added to the extract. We saw a similar result with inhibition of Mps1 as we did with depletion, complete loss of Bub1 (Figure R26a) but not of Sgo1 (Figure R26b). As reversine has also been shown to inhibit Aurora B at some concentrations we checked that pH3 was not lost on chromatin with the treatment (Figure R26c). We would like to combine depletion of Mps1 and inhibition to see if the remaining Sgo1 is due to residual Mps1 activity or there could be an Mps1 independent recruitment.

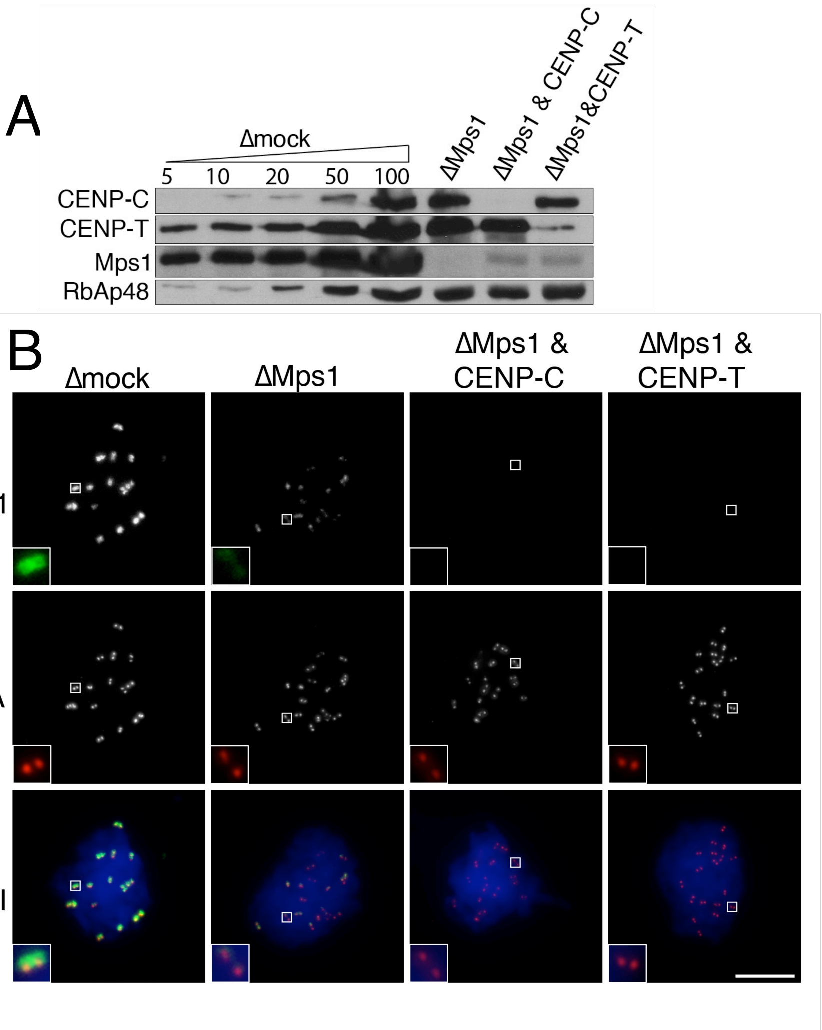


Figure R25. Sgo1 binding to centromeres in Mps1 depleted extracts requires CENP-C and CENP-T. (A) Immunoblot of extract to show efficiency of depletion of Mps1 with either CENP-C or CENP-T. RbAp48, loading control. **(B)** The depleted extract was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Sgo1. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

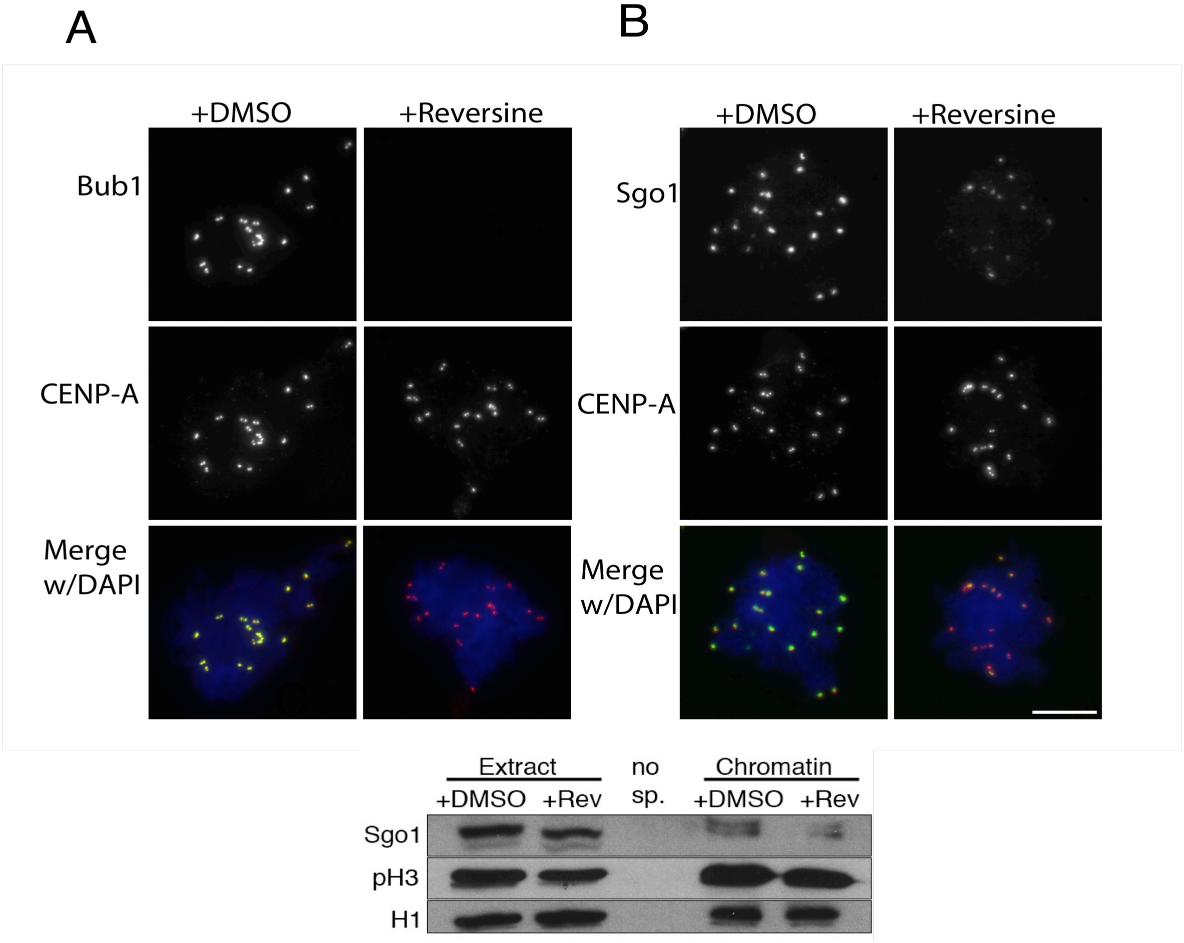


Figure R26. A small amount of Sgo1 is able to bind to centromeres when Mps1 is inhibited chemically. (A) Sperm chromatin was added to interphase extracts, after 90 mins cyclin B was added, to cycle the extract in to mitosis, and they were left for a further 90 mins before fixing. For the final 30 mins 50μM reversine (or DMSO control) was added to the extracts. Chromosomes were fixed and stained for Bub1. Centromeres are marked with CENP-A. DNA was stained with DAPI. **(B)** as in (A) but stained for Sgo1. Scale bar: 10μM. **(C)** immunoblot of chromatin incubated with reversine. H1, loading control.

4.3.4 Role of the CPC in Sgo1 recruitment

Aurora B and the other components of the CPC are known to be important for Bub1 and Sgo1 recruitment. The mechanism for this has not been fully defined. We were interested to see which points in the pathway were affected by depletion of the CPC. We depleted the

CPC from the extract (Figure R27a) and assembled cycled mitotic chromosomes. We saw no effect on either CENP-C (Figure R27b) or CENP-T (Figure R27c).

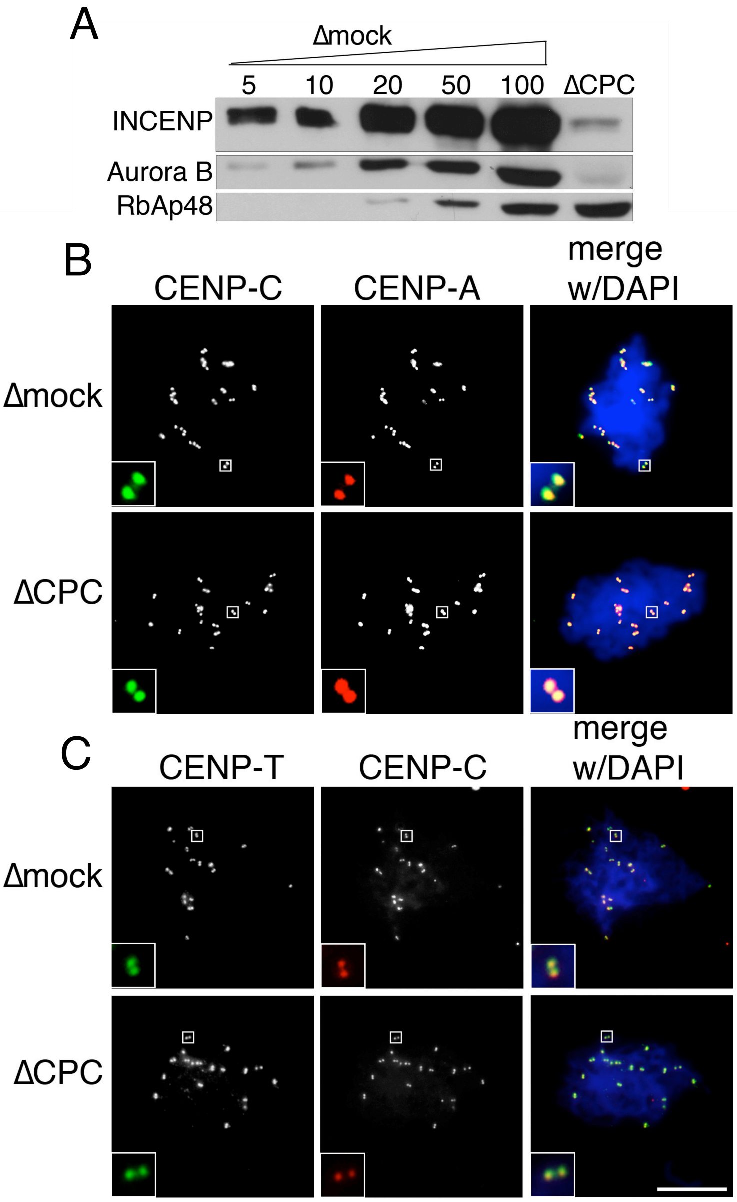


Figure R27. CENP-C and CENP-T do not require the CPC to localise to centromeres. (A) Immunoblot of extract to show efficiency of depletion of CPC components INCENP and Aurora B. RbAp48, loading control. **(B)** The depleted extract was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for CENP-C. Centromeres are marked with CENP-A. DNA was stained with DAPI. **(C)** as in (B) but stained for CENP-T and centromeres were marked with CENP-C. Scale bar: 10μM.

However, we did see a huge decrease in the amount of KMN network proteins Mis12 (Figure R28b) and Ndc80 (Figure R28a) that provide the base for Knl1 and therefore Bub1 binding. These results confirm that CPC depletion causes loss of Mis12 and Ndc80 which has been seen previously in *Xenopus laevis* (Emanuele et al., 2008). For the CPC to localise at centromeres it requires Sgo1 (Rivera and Losada, 2009). When we deplete Sgo1 we don't see an effect on Mis12 or Ndc80 localisation. This suggests that the CPC's role in recruitment of Mis12 and Ndc80 does not require it to accumulate at centromeres. While studying an expandable kinetochore module in Xenopus Wynne and Funabiki saw that although the CPC was essential for expansion it did not need to be localised to the module (Wynne and Funabiki, 2015).

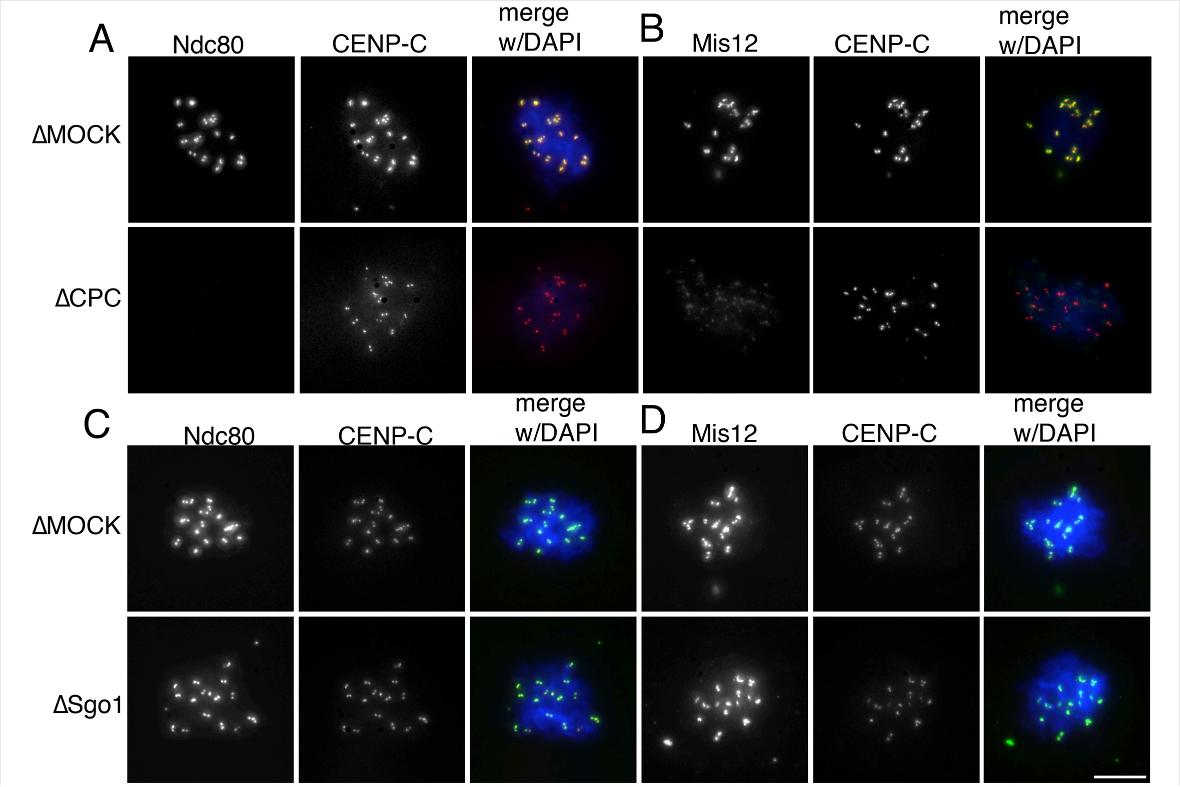


Figure R28. Mis12 and Ndc80 require the CPC but not Sgo1 to localise to kinetochores. Extract depleted of either CPC (A&B) or Sgo1 (C&D) was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for Ndc80 (A&C) or Mis12 (B&D). Centromeres are marked with CENP-C. DNA was stained with DAPI. Scale bar: 10μM.

4.3.5 Bypassing the need for the kinetochore in Sgo1 recruitment

An important question was whether kinetochore and centromere proteins are required for Sgo1 recruitment or whether they are just needed to bring Bub1. So we designed an experiment to recruit the Bub1 kinase domain to centromeres in the absence of kinetochores. CENP-B is commonly used to force target proteins to the centromere however a CENP-B homolog has not been identified in Xenopus. We decided to use the C-terminal fragment of CENP-C which localises to centromeres but does not recruit outer kinetochore proteins (Milks et al., 2009). We created a myc-tagged fusion protein containing the CENP-C domain and the kinase domain from Bub1. This is named CenBub1 from now on, the control myc tagged C-Terminal of CENP-C is known as CenC (Figure R29a).

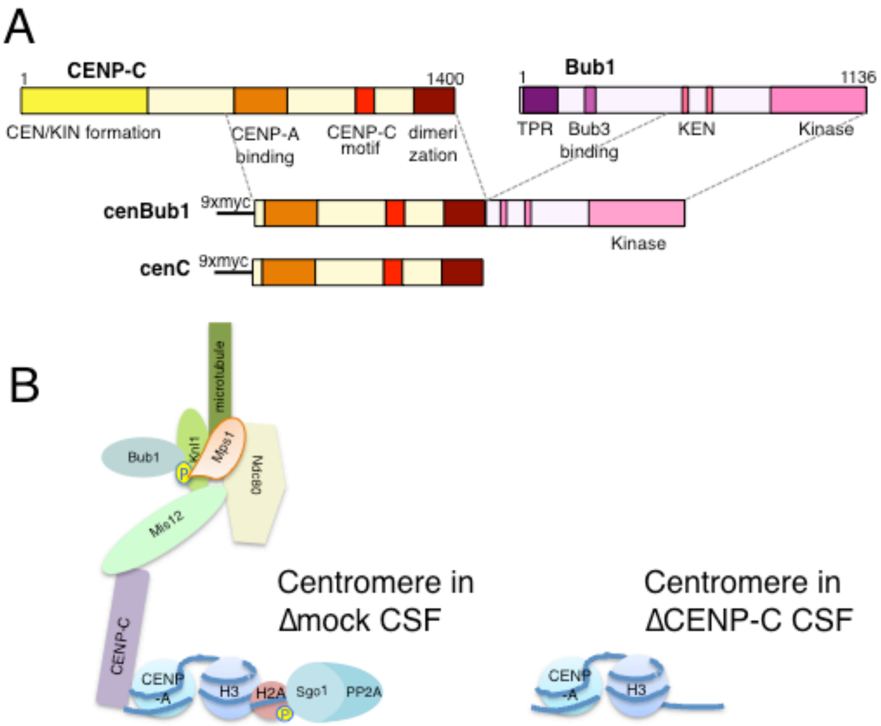


Figure R29. CenBub1 – fusion protein for targeting the kinase domain of Bub1 to centromeres. (A) Schematic representation of construct made to target Bub1 kinase domain to the centromere (CenBub1) and the control fragment of CENP-C (CenC) **(B)** Representation of loss of kinetochore at CSF centromeres with depletion of CENP-C.

Then we used chromosomes assembled in CSF extract depleted of CENP-C to assess if the construct rescued Sgo1 recruitment. In CSF assembled chromosomes only the CENP-

C (not the CENP-T) kinetochore assembly pathway is present so depletion of CENP-C causes complete loss of the kinetochore components as with Sgo1 and Bub1 (Figure 29b). The CENP-C antibody does not recognise the CenC or Cen-Bub1 construct as it is raised against a domain in the N-Terminal of CENP-C. We show the addition in the extract by immunoblot with an antibody against myc (Figure R30a). Then by immunofluorescence with a myc antibody we checked that the cenBub1 and CenC control construct targetted to centromeres in the CSF CENP-C depleted extract (Figure R30b).

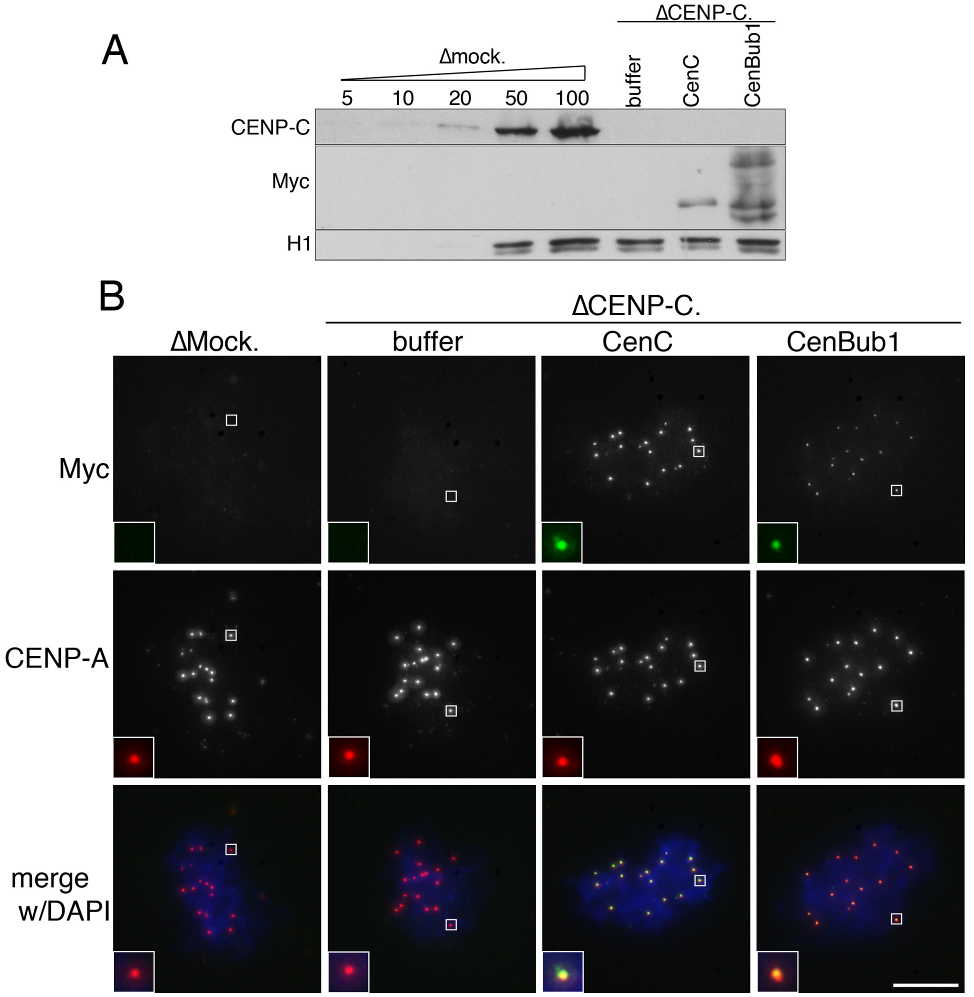


Figure R30. Myc tagged CenBub1 construct localises to centromeres in CENP-C depleted CSF extracts. (A) Immunoblot showing myc-CenC (lane 6) and myc-CenBub1 (lane 7) added to CENP-C depleted extracts. CENP-C antibody does not recognise Cen constructs as it recognises the N-terminal of CENP-C. H1, loading control. (B) Chromosomes assembled in the CSF extracts shown in (A) were fixed and stained with a myc antibody. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

Next we confirmed that our construct did not recruit kinetochore components. After depletion of CENP-C Mis12, Ndc80 and Mps1 are completely removed from the CSF centromeres and neither the CENP-C fragment (CenC) nor the CenBub1 protein rescued them (Figure R31).

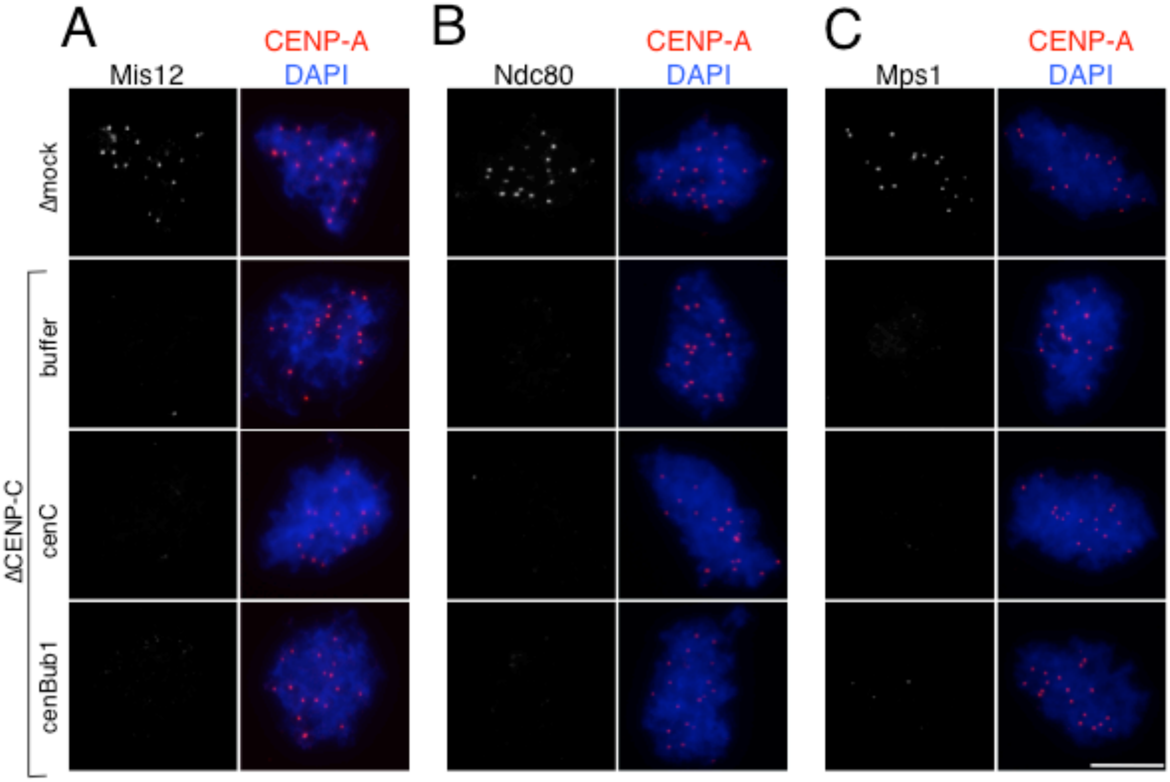


Figure R31. There is no kinetochore at CENP-C depleted CSF centromeres even in the presence of CenBub1. myc-CenC and myc-CenBub1 were added to CENP-C depleted CSF extracts followed by the addition of sperm. Chromosomes were assembled then fixed and stained for Mis12 (A), Ndc80 (B) or Mps1 (C). Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

Then we showed that, despite the absence of the kinetochore, we could rescue Sgo1 (Figure R32a) and pH2A (Figure R32b) by tethering the Bub1 kinase domain to the centromere. Thus suggesting that the essential role of CENP-C and CENP-T is to provide the base for outer kinetochore protein recruitment and therefore Bub1 binding. If they also play another role it is not absolutely required.

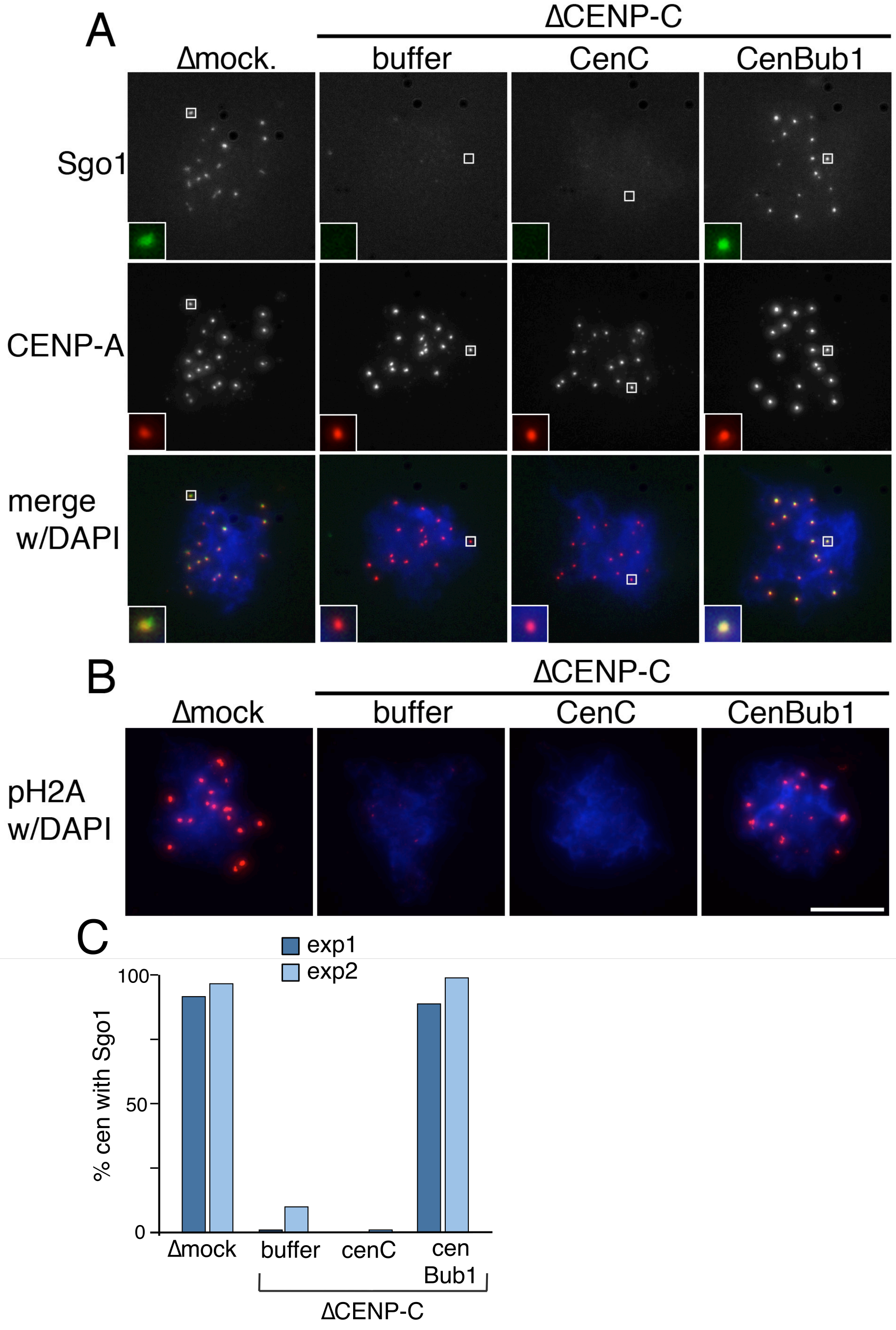


Figure R32. Tethering Bub1 to the centromere in the absence of a kinetochore is sufficient to recruit Sgo1. myc-CenC and myc-CenBub1 were added to CENP-C depleted CSF extracts followed by the addition of sperm. Chromosomes were assembled then fixed and stained for Sgo1 (A) or pH2A (B) Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM. (C) Quantification of the percentage of centromeres with a Sgo1 signal n= >120 centromeres per experiment.

With the rescue of Sgo1 we also rescue the CPC (shown by INCENP staining)(Figure R33). It is known that Sgo1 interacts with the borealin subunit of the CPC. We would like to perform a further experiment were we chemically inhibit aurora B in this system. We could see if the CPC kinase activity is required for Sgo1 rescue or whether its main function in this pathway is in recruitment of Bub1.

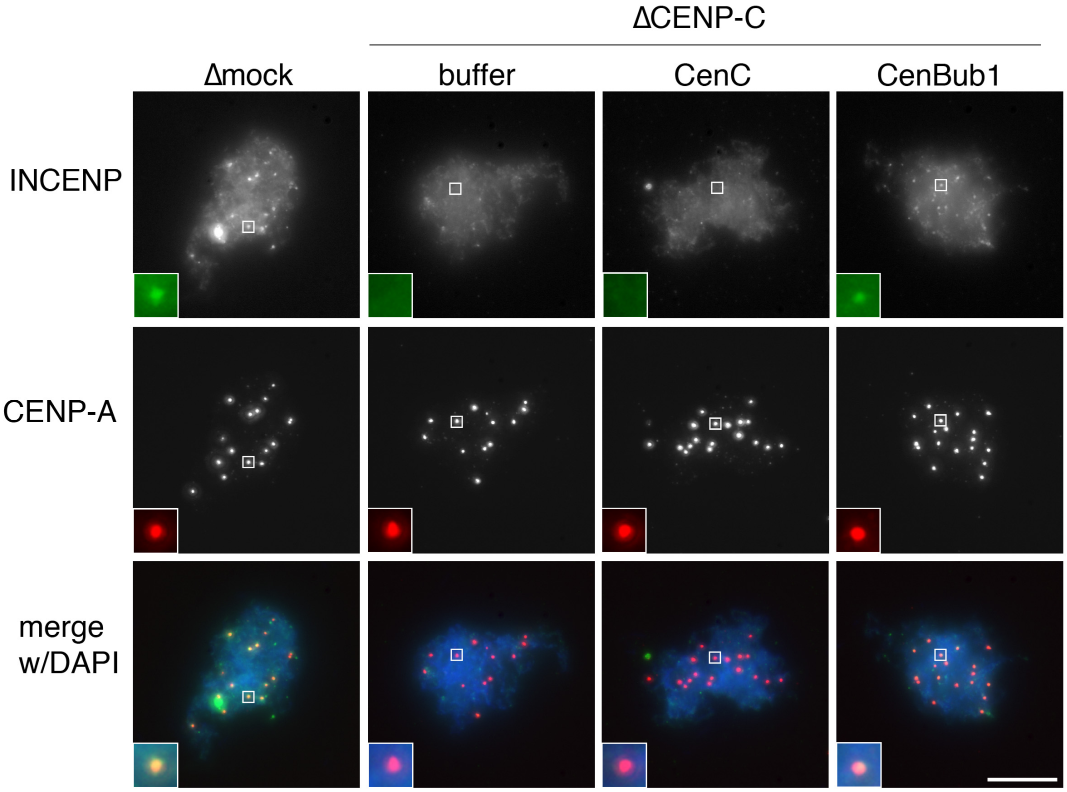


Figure R33. Tethering Bub1 to the centromere in the absence of a kinetochore can recruit the CPC. myc-CenC and myc-CenBub1 were added to CENP-C depleted CSF extracts followed by the addition of sperm. Chromosomes were assembled then fixed and stained for INCENP. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

4.4 Interplay of kinetochore assembly with centromeric cohesion

4.4.1 Role of Mps1 in centromeric cohesion

We previously published that Sgo1 depletion causes an increase in the distance between centromeres in the extract (Rivera and Losada, 2009). This defect was clearly seen in Bub1 depleted extracts but we did not see much of a cohesion defect in Mps1 depleted extracts (Figure R34). Thus suggesting that the small amount of Sgo1 remaining at centromeres in Mps1 depleted extracts (Figure R23) is sufficient for the maintenance of cohesion.

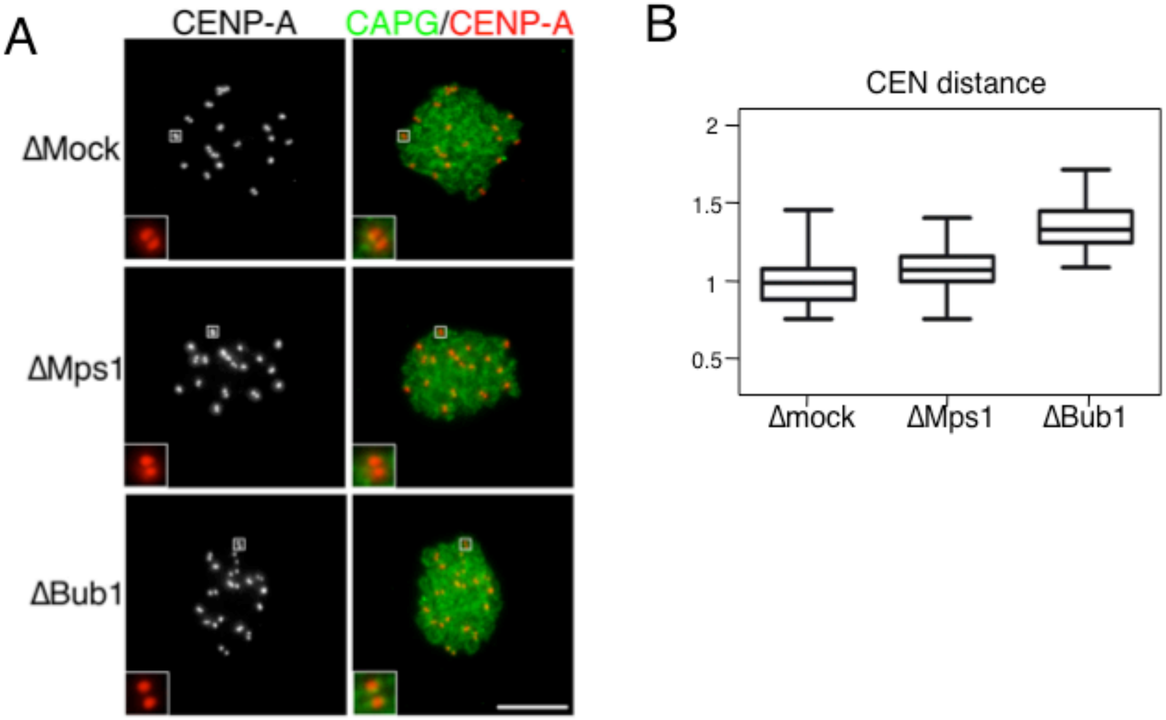


Figure R34. Depletion of Mps1 does not cause a cohesion defect. (A) Extract depleted of Mps1 or Bub1 was converted to interphase, then sperm was added and left 90 mins before adding CSF extract to cycle to mitosis for a further 90 mins. Chromosomes were fixed and stained for CENP-A and condensin subunit CAP-G to stain the chromosome axis. Scale bar: 10μM. **(B)** Box and whisker plot to show distance between centromeres in comparison to the control. For control and Mps1 depletion an average was taken from more than 85 nuclei across 3 different experiments. The Bub1 depletion measurements are from 34 nuclei in one experiment.

4.4.2 Role of CENP-C in centromeric cohesion

We have seen that depletion of either CENP-C or CENP-T has a similar effect on the amount of Sgo1 at centromeres (Figure R17). However, the effect of CENP-C depletion on centromeric cohesion appeared to be far more dramatic than the effect of CENP-T depletion (Figure R35). This suggested that CENP-C might play another role in regulation of cohesion beyond Sgo1 recruitment.

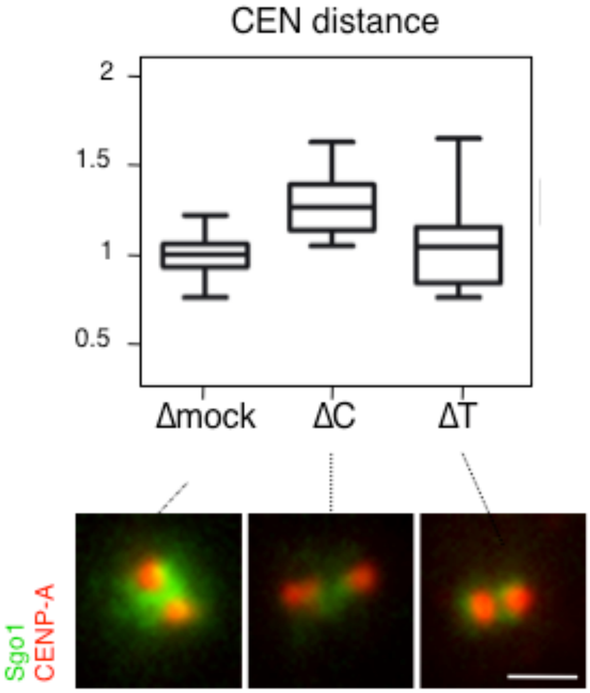


Figure R35. CENP-C is required for cohesion. Distance between sister centromeres (average per chromosome mass) measured in mitotic chromosomes assembled in mock (*n*= 46), CENP-C (n= 33) and CENP-T depleted extracts *(*n= 37) relative to the mean distance observed in the control chromosomes. Data gained from 3 independent experiments. Examples of a single centromere pair stained with CENP-A and Sgo1 are shown below the graph for each condition. Scale bar: 1 *μ*m

One possible reason for the defect could be a reduction in the ability of cohesin to bind to centromeres when CENP-C is depleted. We checked chromatin assembled in extracts depleted of CENP-C or CENP-T and saw no difference in levels of cohesin by immunoblot (Figure R36a). We also checked by immunofluorescence and saw no difference, although quantification was not possible (Figure R36b).

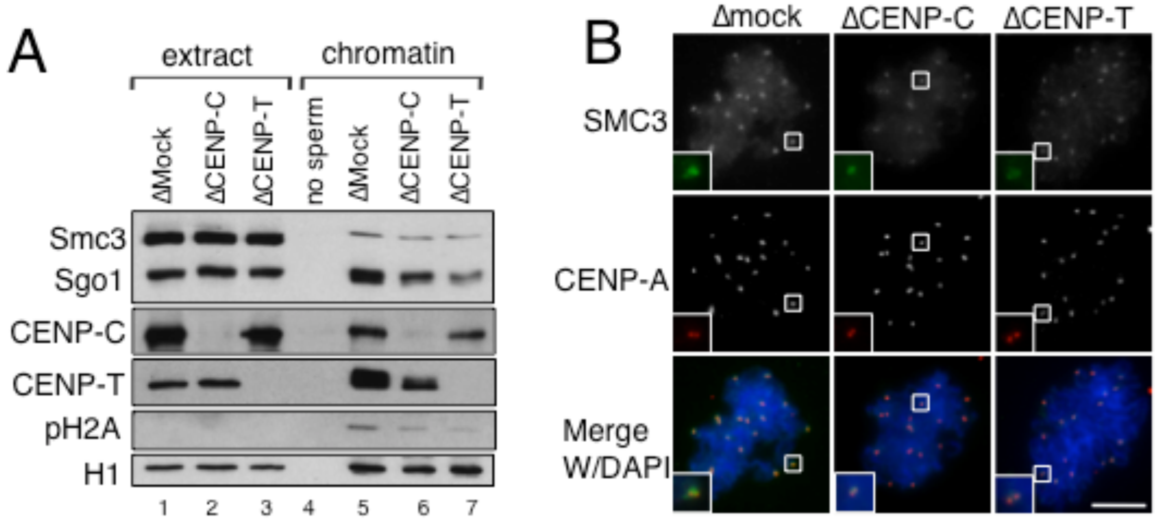


Figure R36. There is no difference in the levels of cohesin at centromeres in the absence of CENP-C or CENP-T. (A) Immunoblot analysis of chromatin fractions from cycled mitotic chromosomes assembled in the indicated extracts and a mock assembly reaction without sperm as control (lane 4). Histone H1 was used as loading control. **(B)** Mitotic chromosomes assembled in extracts lacking CENP-C or CENP-T, or mock depleted, and stained with antibodies against cohesin Smc3 subunit. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

Since it is unlikely reduced cohesin binding is the reason for the cohesion defect in CENP-C depleted extracts we had to look for other possibilities. We observed by immunofluorescence that CENP-A levels at centromeres were reduced in the CENP-C depleted chromosomes but not the CENP-T (Figure R37). This could mean that CENP-A levels are important for cohesion. We would like to assess whether altering the levels of CENP-A at the centromere through HJURP, the CENP-A loading protein, or CENP-A depletion can cause cohesion defects.

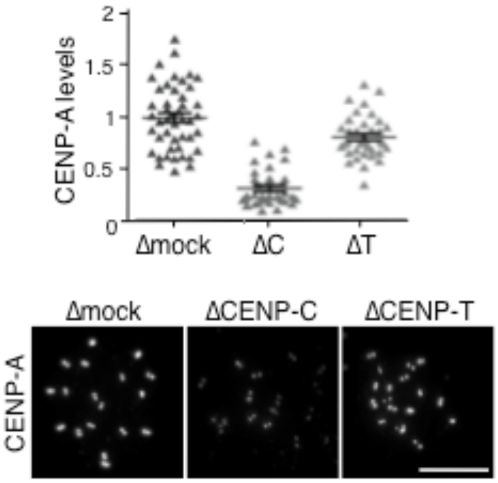


Figure R37. CENP-C is required for CENP-A maintenance. Levels of CENP-A were measured in mitotic chromosomes assembled in extracts lacking CENP-C or CENP-T, or mock depleted, and stained with a CENP-A antibody. DNA was stained with DAPI. Scale bar: 10μM. n= >32 nuclei from 3 separate experiments.

We were interested to know whether we could also see the effect on cohesion in human cells. We transfected cells with siRNA against CENP-C or CENP-T. Unlike in the extract we saw a much bigger effect on Sgo1 in CENP-C depleted cells than in CENP-T depleted cells (Figure R38). However this could be because CENP-T depletion was not as efficient as CENP-C (Figure R38a).

When we measured the distance between centromeres in the siCENP-C condition we saw a possible increase in the average distance between centromere (Figure R39). The conditions for this experiment would need to be improved so that we can be sure the cells we measuring are those with CENP-C knocked down. We could try a plasmid that co-expresses shRNA against CENP-C and a GFP tag. However a defect in cohesion could also be due to the greater effect on Sgo1 levels seen in this system.

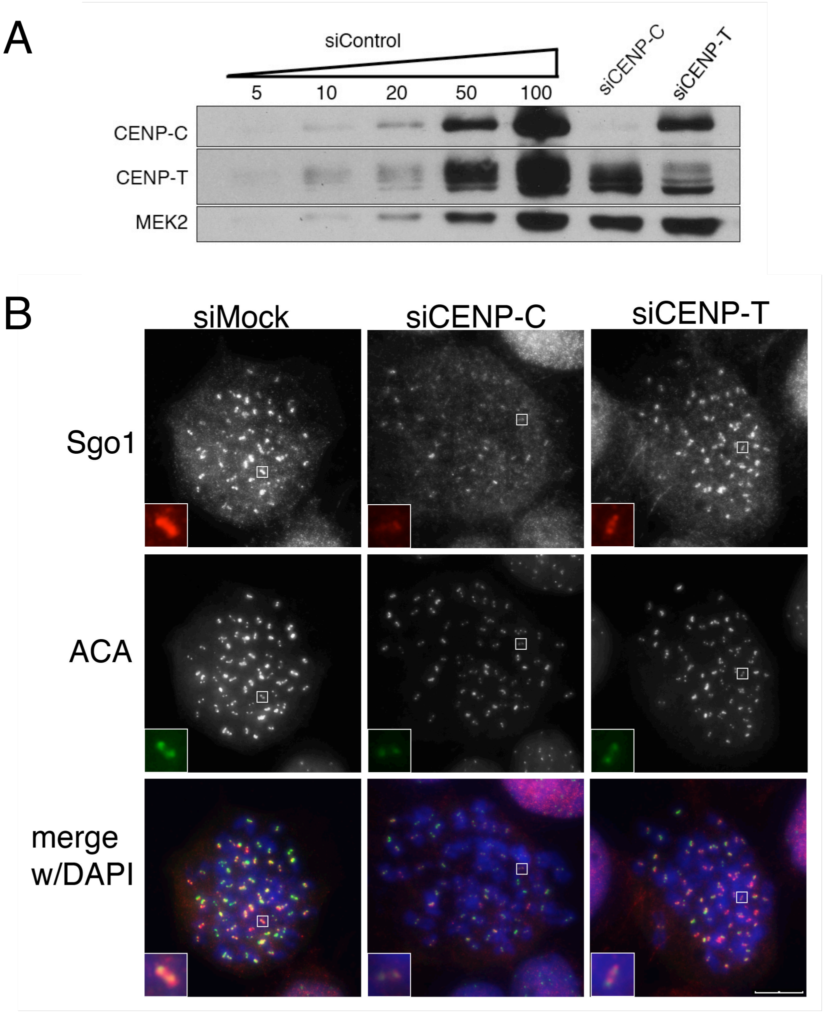


Figure R38. In HeLa cells Sgo1 is more affected by depletion of CENP-C than CENP-T. (A) Immunoblot analysis of total cell extracts from HeLa cells 24 hours after a two hit transfection with CENP-C or CENP-T siRNA. MEK2, loading control. **(B)** Cells were incubated for 2 hours with nocodazole before a 30 min hypotonic treatment, then fixed and stained for Sgo1 and anti centromere antibody (ACA).

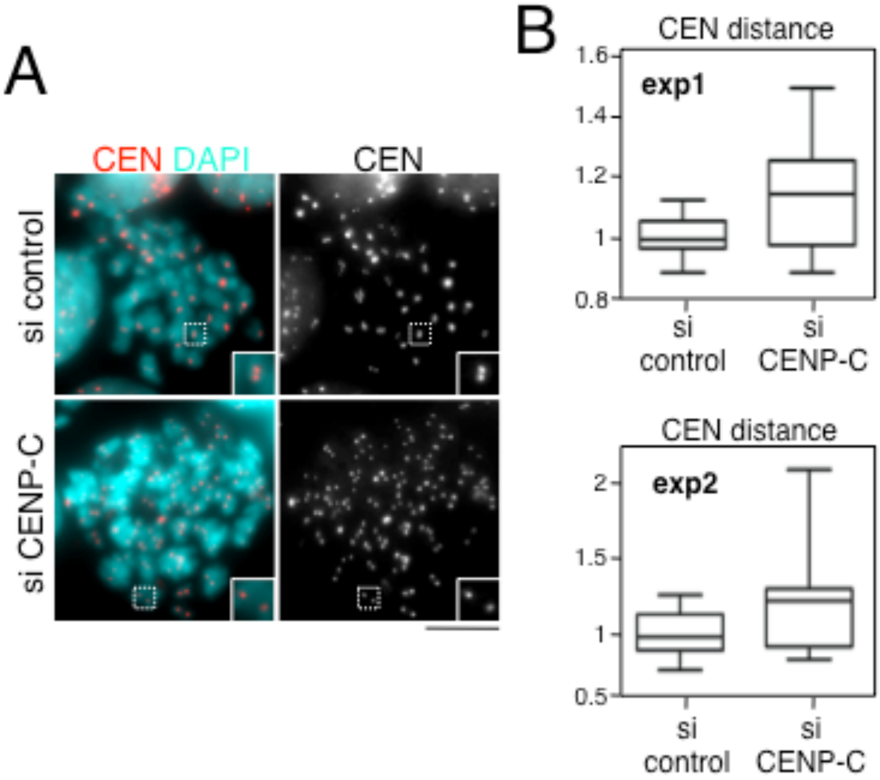


Figure R39. Depletion of CENP-C in HeLa cells may cause increased separation of centromeres. (A) Representative examples of mitotic chromosomes from control cells and cells transfected with CENP-C siRNA stained with DAPI (blue) and anti-centromere antibody (CEN, red). Cells were incubated in nocodazole for 2 hours before a 30 min hypotonic treatment and fixation. Scale bar, 10 μm. **(B)** Distance between sister centromeres (average per nucleus) after siRNA treatment in two independent experiments. Chromosomes from at least 15 cells were measured per condition in each experiment.

4.5 Centromere and kinetochore assembly's role in condensin II recruitment to the centromere

Condensins are from the same family of SMC proteins as cohesin. However their main function is when cells enter mitosis and most of cohesin is released. They aid the condensation of chromatin to mitotic chromosomes. There are two main forms condensin I and condensin II. In the Xenopus egg extract condensin I is much more abundant and appears to play a more important role. Despite this, a population of condensin II accumulates at the centromere as in other models. The function of this population is not clear. Many of the proteins associated with shugoshin and its recruitment have been associated with the recruitment of condensin II. We thought it was important to check the involvement of the Sgo1 recruitment pathway and condensin II in the xenopus system. We used immunofluorescence of cycled mitotic chromosomes as these appear to be more physiological relevant than CSF chromosomes, although we also see accumulation at CSF centromeres. In this case we did not look by immunoblot as condensin II is all over chromosomes in mitosis and we are interested specifically in the population concentrated at centromeres. Both shugoshin proteins have been shown to be important in condensin II recruitment. Despite this, depletion of either Sgo1 (Figure R40) or Sgo2 (Figure R41) did not reduce recruitment of condensin II to centromeres. Both Sgo1 and Sgo2 require Bub1 to be recruited to the centromere. We depleted Bub1 kinase and saw no change in recruitment of condensin II (CAP-H2) by immunofluorescence. This shows Bub1 is not required and also reduces the possibility that one shugoshin compensates for the other in the Sgo1 or Sgo2 depletions.

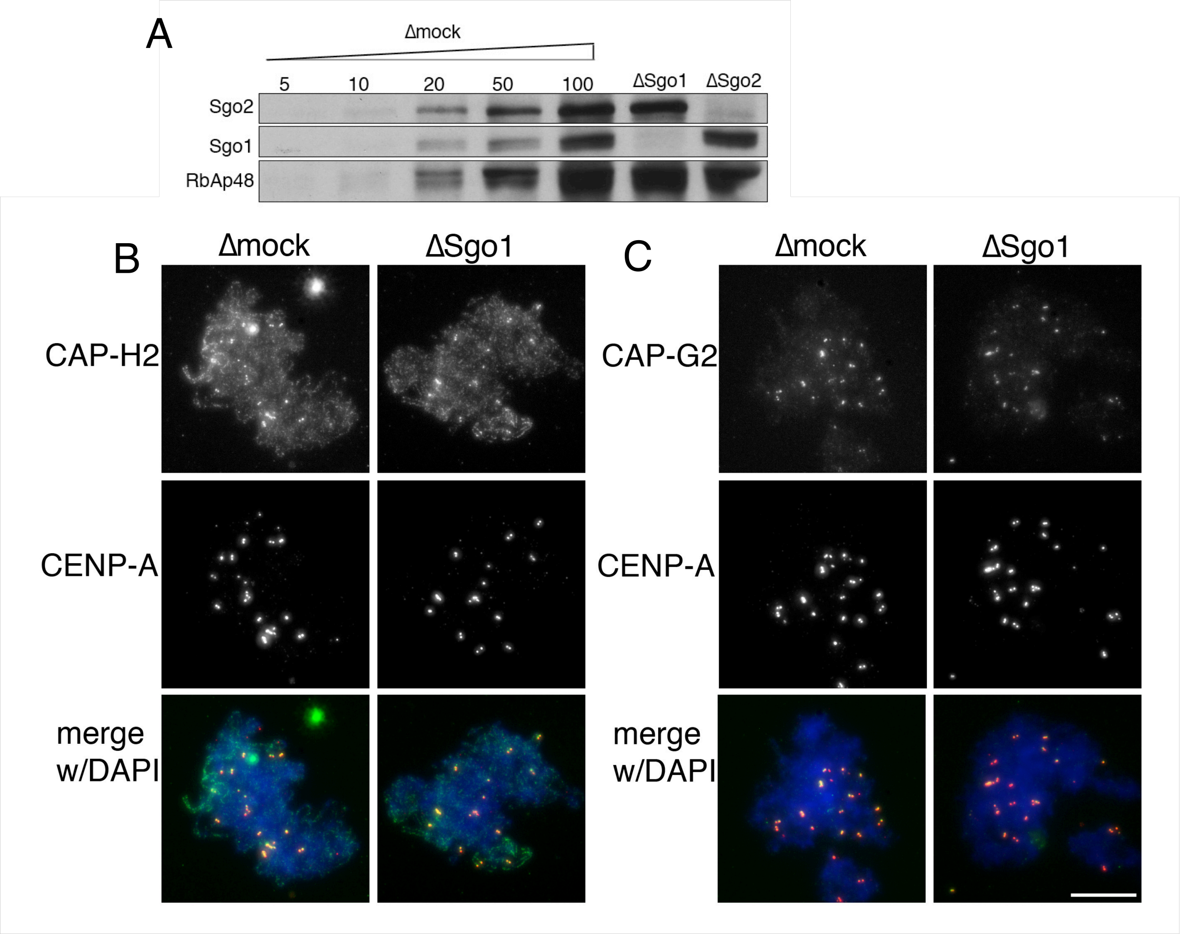


Figure R40. Condensin II can be recruited to centromeres in the absence of Sgo1. (A) Immunoblot to show efficiency of depletion of Sgo1 and Sgo2 from extract. RbAp48, loading control. **(B)** Cycled mitotic chromosomes assembled in extract depleted of Sgo1 stained for condensin II subunit CAP-H2. Centromeres are marked with CENP-A. DNA was stained with DAPI. **(C)** As in (B) but stained for condensin II subunit CAP-G2. Scale bar: 10μM.

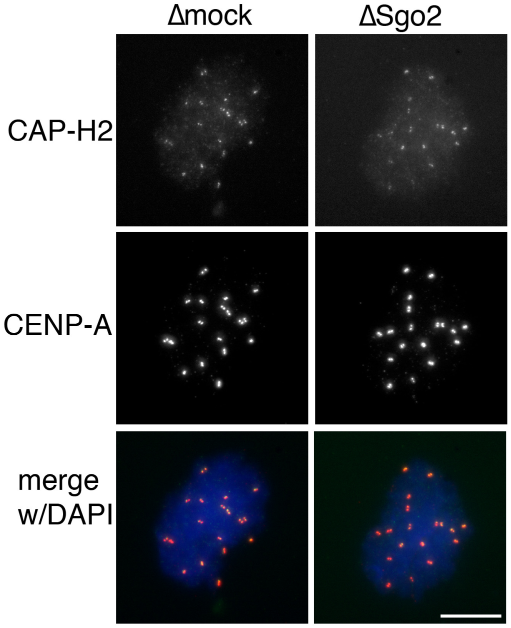


Figure R41. Condensin II can be recruited to centromeres in the absence of Sgo2. Cycled mitotic chromosomes assembled in extract depleted of Sgo1 stained for condensin II subunit CAP-H2. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

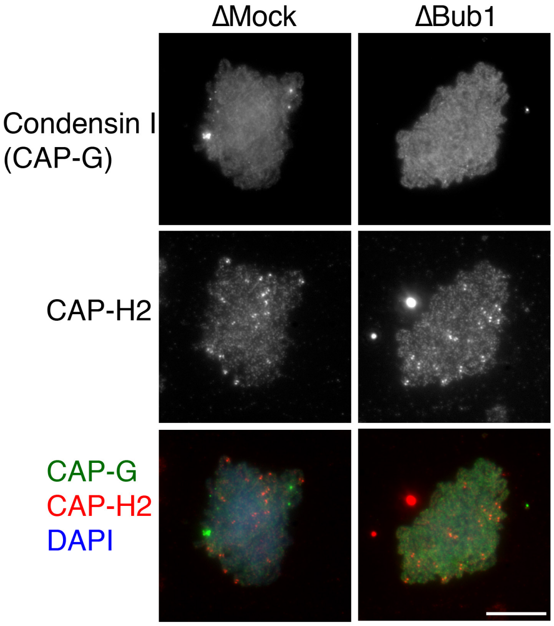


Figure R42. Bub1 is not required for condensin II recruitment. Cycled mitotic chromosomes assembled in extract depleted of Bub1 stained for condensin II subunit CAP-H2, and condensin I subunit CAP-G. DNA was stained with DAPI. Scale bar: 10μM.

Mps1 has also been described as being important in recruitment of condensin II to mitotic chromosomes in human cells (Kagami et al., 2014). When we assembled chromosomes in Mps1 depleted extracts we failed to see a decrease in the recruitment of condensin II to centromeres.

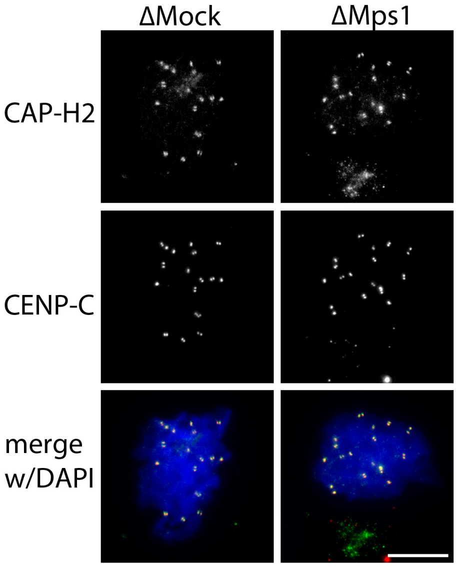


Figure R43. Mps1 is not required for condensin II recruitment to the centromere. Cycled mitotic chromosomes assembled in extract depleted of Mps1 stained for condensin II subunit CAP-H2. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

As has been described in other systems, the CPC was required for condensin II accumulation at centromeres (Figure R44). A small amount of condensin II could still be detected at centromeres but much less than in the control condition. Depletion of the CPC is good with less than 5% remaining (Figure 27a) however a small amount of kinase can be sufficient to catalyse phosphorylation and the remaining condensin II could be due to this. Sgo1 is required for CPC to localise at centromeres but Sgo1 depletion does not disrupt condensin II recruitment to the centromere. This suggests that the CPC function in condensin II recruitment may not be reliant on centromere accumulation.

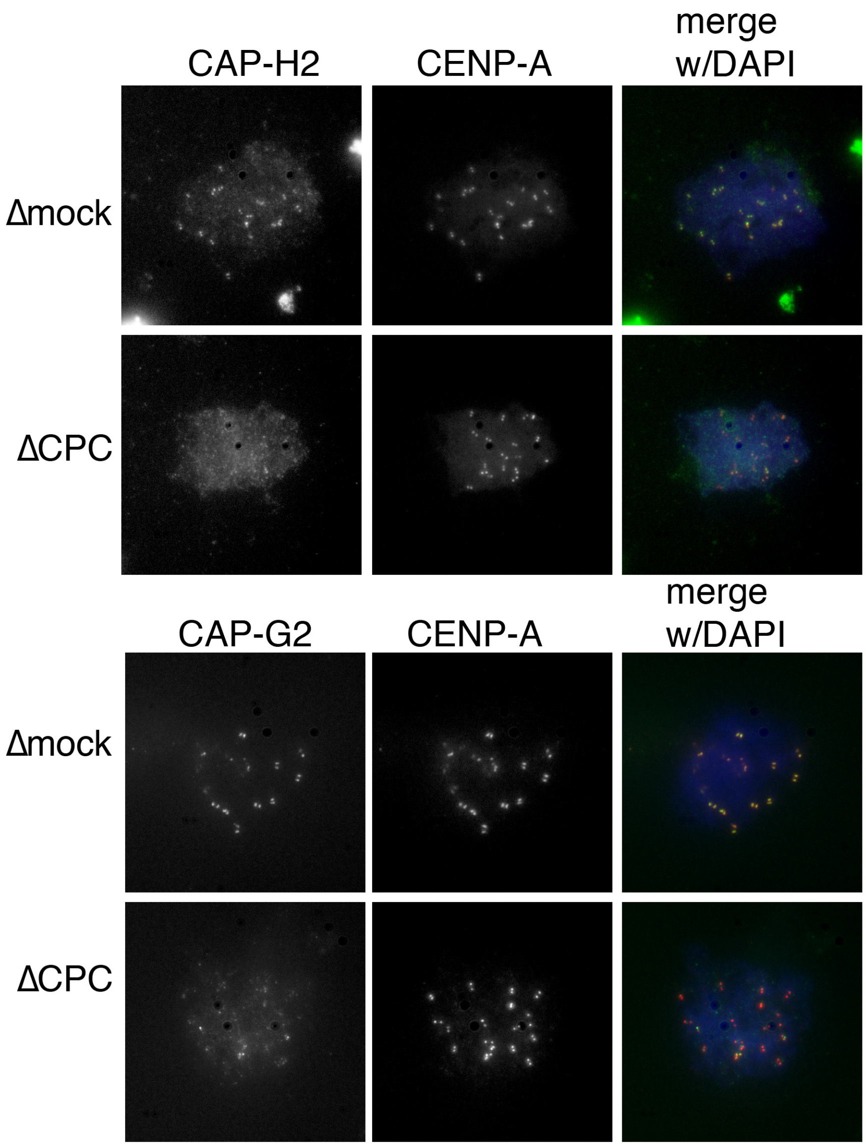


Figure R44. Depletion of the CPC reduces condensin II accumulation at the centromere. (A) Cycled mitotic chromosomes assembled in extract depleted of the CPC stained for condensin II subunit CAP-H2. Centromeres are marked with CENP-A. DNA was stained with DAPI. **(B)** As in (A) but stained for condensin II subunit CAP-G2. Scale bar: 10μM.

We purified condensin II from the egg extracts and performed an in vitro phosphorylation reaction with immunoprecipitated CPC/Aurora B. We were unable to identify a difference in the phosphorylation pattern of condensin II incubated with CPC from interphase and mitotic extracts (data not shown). However with our purifications of condensin II we were also not clearly able to identify the different subunits of condensin II on a silver stained gel.

We need to find an improved way of purifying condensin II from the extract or generate recombinant protein of the three condensin II specific subunits.

Although CENP-I has been implicated, the centromere component requirements for condensin II recruitment are not well defined. We showed that CENP-C was required whereas CENP-T, CENP-N and CENP-K were not. We would like to look at which domains of CENP-C are important for condensin II recruitment. As a starting point we can check using our C-terminal of CENP-C construct, that targets to the centromere, whether the N-terminal of CENP-C is required or not.

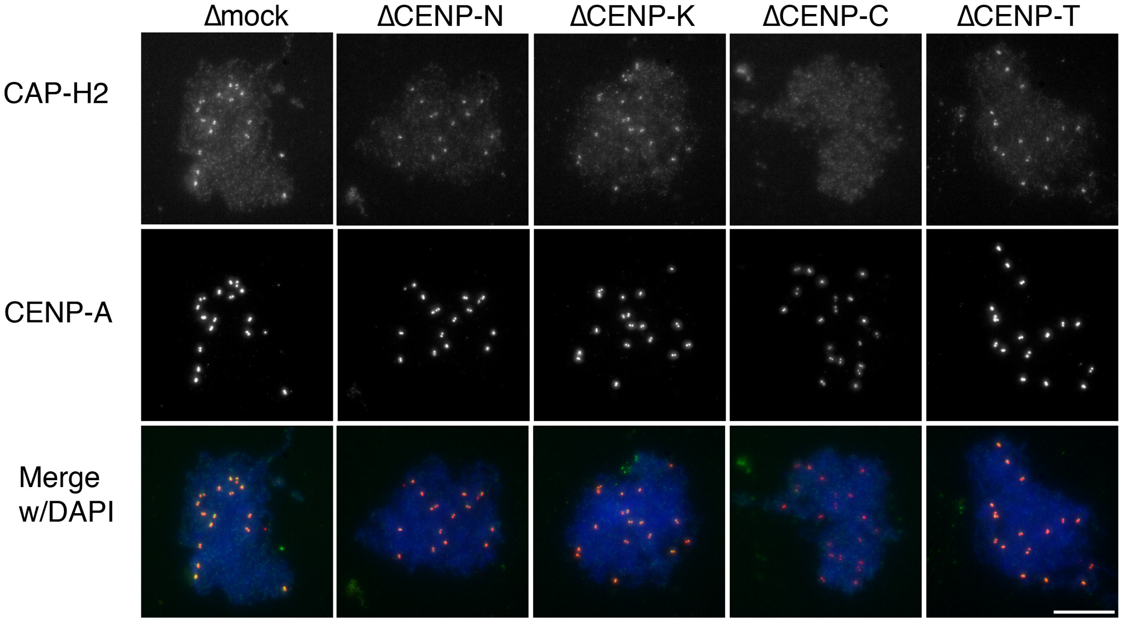


Figure R45. CENPC is required for condensin II recruitment to the centromere while CENP-T, CENP-N and CENP-K are dispensable. Interphase extract was depleted of CENP-N, CENP-K, CENP-C or CENP-T and sperm chromatin added, after 90 mins cyclin was added, to cycle the extract in to mitosis, and they were left for a further 90 mins before fixing. Chromosomes were stained for condensin II subunit CAP-H2. Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

We wanted to see if we could detect a direct interaction between CENP-C and condensin II in the extract. We immunoprecipitated two of the condensin II subunits CAP-H2 and CAP-D3 and saw a possible interaction of endogenous CENP-C in undepleted extract (Figure 46 lanes 7 & 8). As the band was weak to validate if this was truly an interaction with CENP-C we performed the same immunoprecipitation in CENP-C depleted extract (Figure 46 lanes 9&10). When CENP-C was not present in the extract the band no longer appeared in the condensin II IP suggesting it is CENP-C and we can detect an interaction between condensin II and CENP-C in the soluble mitotic extract.

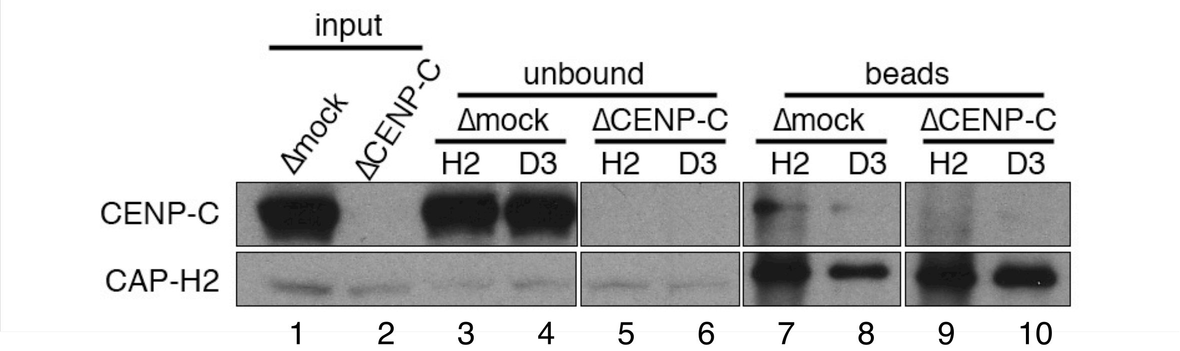


Figure R46. CENP-C and condensin II may interact directly. High-speed CSF extracts were depleted using rabbit IgG or a CENP-C antibody. Condensin II subunits CAP-H2 (lane 7 & 9) and CAP-D3 (lane 8 & 10) were immunoprecipitated from both extracts and analysed by immunoblot. Unbound fractions (lanes 4 – 6) and input extract (lane 1 & 2) were also analysed.

We would like to generate recombinant CENP-C protein to see if this interacts with condensin II that has been immunoprecipitated from the extract. If we manage to confirm the interaction in this manner we would like to further investigate this interaction. We will generate a number of truncated CENP-C proteins to decipher which part of CENP-C condensin II is interacting with. If this work is successful we would then try to identify which subunit or subunits of condensin II it interacts with and where.

4.6 Role of condensin II at the centromere

The role of condensin II at the centromere has not been clearly defined and this is something we are interested in investigating further. Condensin proteins have also been linked with regulation of cohesion (Lam et al., 2006). As condensin II is concentrated at the centromeres we wondered if it plays a more specific role in centromeric cohesion. So we checked if removal of condensin II affected the recruitment of Shugoshin. We depleted CAP-D3 from the extract and checked the levels of Bub1, pH2A, Sgo1 and Sgo2 (Figure R47). We did not identify any clear defects suggesting that condensin II is not involved in protecting cohesion through recruitment of shugoshin. We would like to measure to see if there is an increase in the distance between centromeres with removal of condensin II that would suggest a role in cohesion.

It was previously seen in the lab that a defect in CENP-A loading occurs in xenopus egg extracts when condensin II is depleted. A defect in CENP-A loading has also been seen with depletion of the SMC2 subunit of both condensin I and II in human cells (Samoshkin et al., 2009). We wanted to see if we could see a defect in CENP-A in human cells with depletion of just condensin II. We depleted the CAP-D3 subunit of condensin II by siRNA in 293T cells that express GFP-CENP-A on addition of doxycycline. We saw no significant effect on levels of GFP-CENP-A at centromeres. A more sensitive way to look specifically at CENP-A loading in human cells would be to use a pulse-chase imaging system (Bodor et al., 2013) and we could try this method. The recruitment of condensin II and its function at centromeres are questions that remain unclear and we wish to investigate them both further in the future.

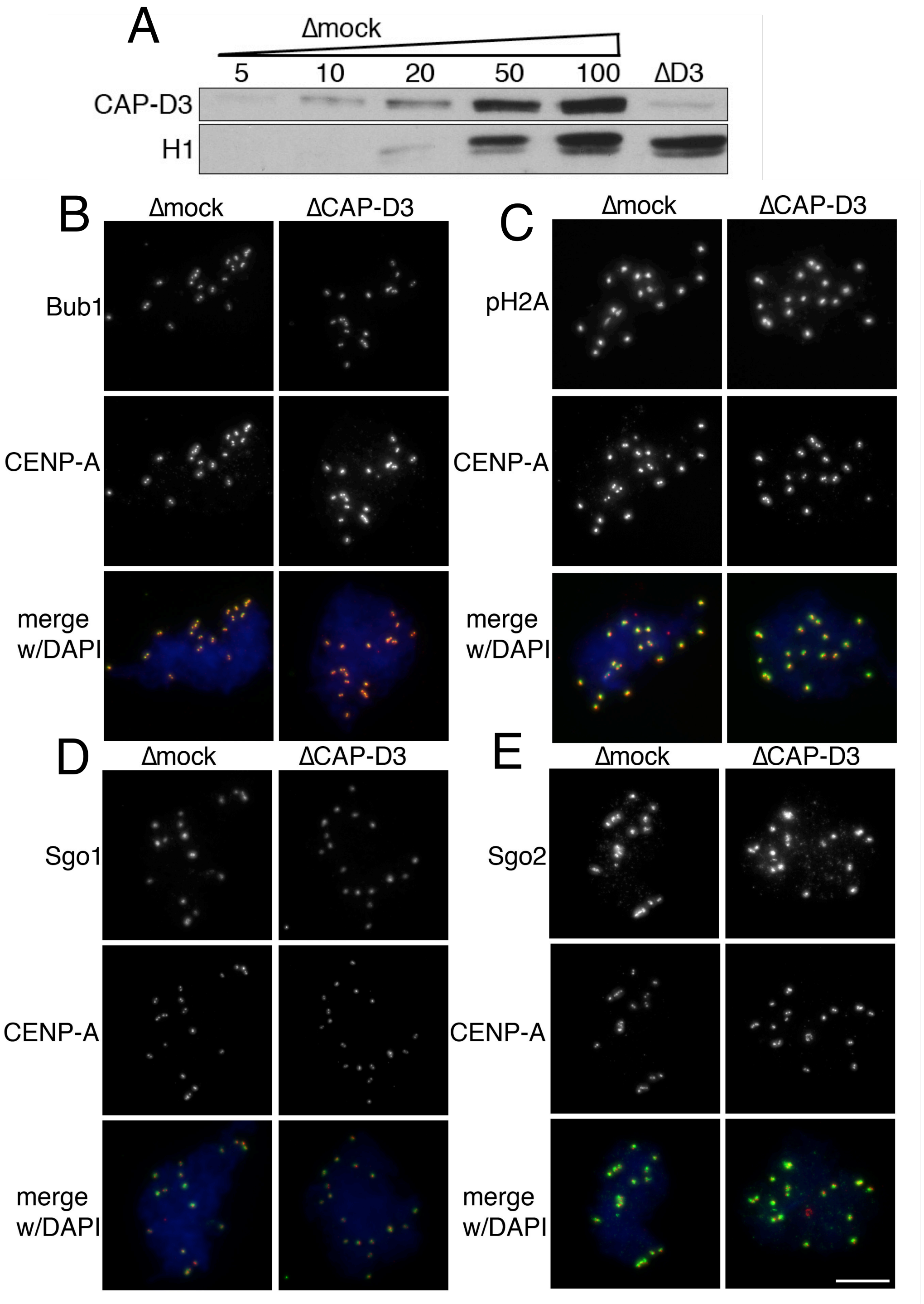


Figure R47. Condensin II is not required for recruitment of shugoshin. (A) Immunoblot to show efficiency of depletion of condensin II subunit CAP-D3 from extract. H1, loading control. Cycled mitotic chromosomes assembled in extract depleted of CAP-D3 stained for Bub1 (B), pH2a (C), Sgo1 (D) or Sgo2 (E). Centromeres are marked with CENP-A. DNA was stained with DAPI. Scale bar: 10μM.

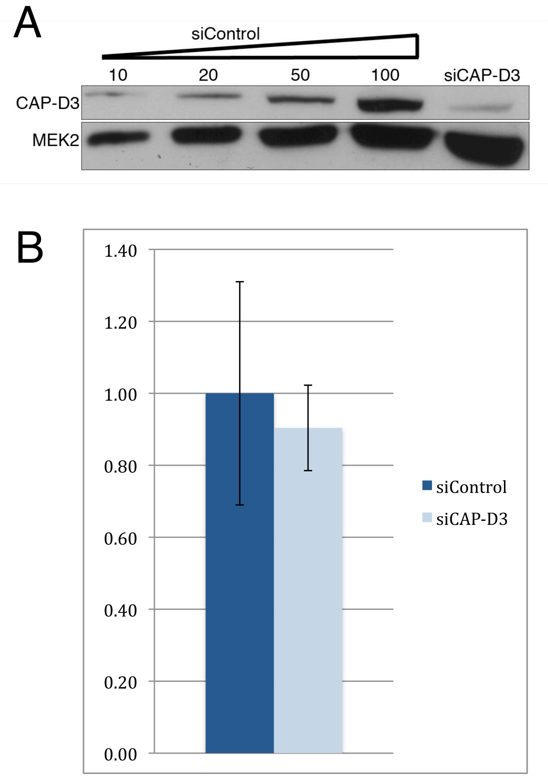


Figure R48. Condensin II depletion does not affect centromeric CENP-A levels in human cells. (A) Immunoblot analysis of total cell extracts from 293T cells that express GFP-CENP-A on addition of doxycycline, cells 48 hours after a two hit transfection with siRNA against condensin II subunit CAP-D3. MEK2, loading control. (B) Quantification of GFP foci expressed as percentage of control condition. Mock (n= 8123) siCAP-D3 (n= 6202).

5. Discussion

5. Discussion

5.1 Characterisation of CSF and cycled mitotic centromeres

Differences in binding of kinetochore and SAC proteins have been seen between CSF and cycled mitotic centromeres (Maresca and Heald, 2006, Boyarchuk et al., 2007). Here we characterised binding of CCAN proteins and saw that only CENP-C not CENP-T, CENP-N or CENP-K localised to CSF centromeres. There is less Ndc80 on CSF kinetochores (Maresca and Heald, 2006), as we show and has been seen before CENP-T recruits Ndc80 to centromeres. Thus the higher levels on cycled mitotic centromeres could easily be explained by the inability of CENP-T to bind to CSF centromeres. We can not rule out that there is some binding of CENP-N, CENP-K and CENP-T at CSF centromeres but if there is it is much less efficient than cycled centromeres. We would like to understand why the centromere proteins don’t bind during CSF. CENP-N is known to interact directly with CENP-A nucleosomes (Carroll et al., 2009) so one might expect that it would be able to recognise the CENP-A at CSF centromeres. One study suggests that CENP-N can only recognise CENP-A during interphase due to the binding site being hidden by a closed chromatin conformation (Fang et al, 2015). So it could be that the decondensation during interphase is required for CENP-N binding to CENP-A. As we have shown CENP-K and CENP-T have a large degree of dependency on CENP-N. We could try to artificially target CENP-N to CSF centromeres to see if we then recruit CENP-K and CENP-T.

5.2 Hierarchy of CCAN subcomplexes

An important result from out study of interdependencies is that CENP-T is not completely dependent on CENP-C for localisation to the centromere. We clearly show in our system that CENP-T can bind independently of CENP-C albeit at a reduced level. Others working in the Xenopus egg extract cell free system have now also seen this (Wynne and Funabiki, 2015). The data regarding dependence of CENP-T on CENP-C has continued to be variable throughout studies in human cell lines. In conventional siRNA studies CENP-T is reduced on centromeres but usually some remains. The Mussachio group report reduction of CENP-T to between 20% and 25% with depletion of CENP-C inferring that indicates complete dependence (Basilico et al., 2014, Klare et al., 2015). While the Salmon group have performed kinetochore architecture studies in CENP-C depleted cells, in conditions were they see no CENP-C on centromeres they see only partially reduced CENP H-I and CENP-T (Suzuki et al., 2014). The latest data from CRISPR Cas9 conditional knock outs in human cell lines suggest that all three of the other complexes are downstream of CENP-C and dependent on each other but this is after five days of doxycycline treatment to knock out CENP-C (McKinley et al., 2015). This indicates that all the complexes have a degree of interdependency on each other, downstream of CENP-C, over several cell cycles but our results show the immediate effect on assembly. In the *Xenopus laevis* egg extract system we have complete depletion of the protein before assembly of the CCAN or kinetochore. Therefore, we may expect to see differences to conventional depletion methods i.e siRNA and conditional knockouts. In an effort to avoid this caveat of gradual depletion some labs have recently been developing the auxin inducible degron system as a way to look at effects of rapid depletion of CENP proteins (Holland et al., 2012). Although up to now there is no publication using this system to degrade CENP-C an undergraduate thesis from the Straight lab has shown when CENP-C is degraded rapidly CENP-T is not lost from centromeres (Cordova, 2015). It will be interesting to see if this result is supported in a peer-reviewed article in the near future. We would suggest that CENP-T binds to centromeres independently of CENP-C but then CENP-C is required to maintain CENP-T at centromeres. This may be because the other CCAN complexes CENP-L-N, CENP-H-I-K-M have a degree of dependency on CENP-C and CENP-T has a degree of dependency on them.

Our results showed that CENP-T is more dependent on CENP-N and CENP-K than CENP-C. A recent study of the proteome of mitotic chromosomes from chicken cells confirmed results previously seen by immunofluorescence, CENP-C depletion caused reduction but not loss of the other CENP proteins, while depletion of CENP-N had a much stronger effect on CENP-T binding to chromatin (Samejima et al., 2015). It was also recently shown in human cells that Auxin degron degradation of CENP-N and CENP-I results in loss of CENP-T at centromeres in mitosis and interphase (McKinley et al., 2015). We showed that CENP-N and CENP-K have a strong degree of interdependence. This is consistent with what has been seen before in human cells (McClelland et al., 2007). We also identified that CENP-T is not immediately required for binding of CENP-C, CENP-N or CENP-K to centromeres. In some studies, it has been suggested that CENP-T is required for binding of the CENP-H-I-K-M and CENP-N complexes. However, in this case a very recent study using a rapid degradation system also supports our results. Using a chicken DT40 cell auxin degradable CENP-T system CENP-T was rapidly depleted specifically in mitosis. This only caused the loss of the T-W-S-X complex on chromatin none of the other CENP proteins were decreased (Wood et al., 2016). This is consistent with our results that CENP-T is not required for centromere binding of other CENPs.

CENP-C does not require other CCAN proteins to localise to centromeres in mitosis in the Xenopus egg extract. While we see a small decrease in interphase CENP-C is able to bind centromeres in the absence of other CENPs. A difference in CENP-C's dependence on CENP-H-I-K-M complex had been seen between interphase and mitosis had previously been seen in chicken (Kwon et al., 2007). Recently differences in CENP-C's dependency on CENP-N and CENP-I in interphase and mitosis have also been shown in human cells, using a rapid degradation system, CENP-C was reduced in interphase but not mitosis (McKinley et al., 2015). The most striking difference between interphase and mitosis that we saw was CENP-T's dependence on CENP-K. It was much more dependent on the presence of CENP-K in mitosis than interphase. This is not consistent with what was seen in human cells with the rapid degradation of CENP-I specifically in interphase. In that case CENP-T was lost with depletion of CENP-I a different member of the CENP-H-I-K-M complex (McKinley et al., 2015). We should further confirm this result in the Xenopus system by depleting one of the other CENP-H-I-K-M complex members.

It is likely there is a conformational change of the centromere region that occurs between interphase and mitosis that confers changes in dependencies. Understanding this change is an interesting future goal for the field. We consider that our results provide an added degree of understanding to the complicated interdependencies of the CCAN protein network. We do keep in mind that some results may be specific to Xenopus or embryonic systems. However, we believe the emergence of the rapid degradation systems will give us greater understanding as to which aspects are conserved in human cells.

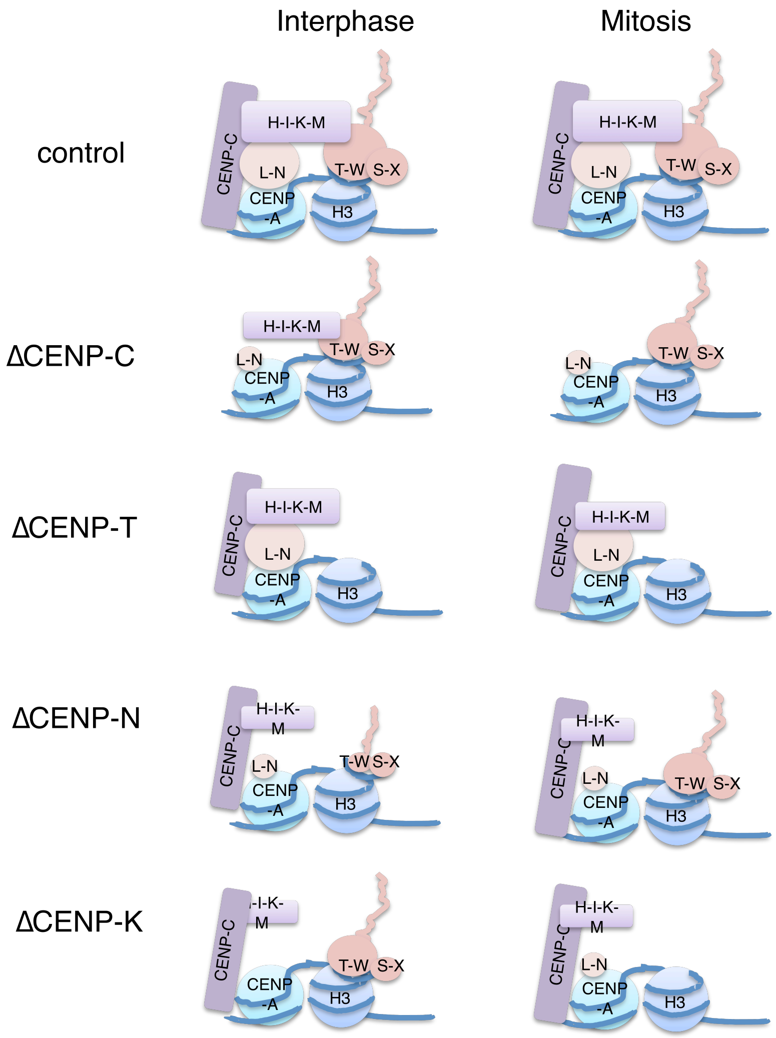


Figure D1 CCAN Subcomplex interdependencies

5.3 Sgo1 recruitment and centromeric cohesion

5.3.1 CENP-C and T pathways

In our system we identify that CENP-T is able to recruit the entire KMN network in the absence of CENP-C. It is already known that CENP-T interacts with and recruits Ndc80 (Nishino et al., 2013). One possibility is that since Mis12 can interact with Ndc80 (Petrovic et al., 2010) the CENP-T recruits Ndc80 and then this recruits Mis12. However it has been suggested that the binding of Mis12 and CENP-T to Ndc80 occurs at the same site and they compete with each other (Malvezzi et al., 2013). Another possibility is that CENP-T directly interacts with Mis12, an interaction has been detected in vitro with recombinant proteins (Gascoigne et al., 2011). This ability of CENP-T means it is able to recruit Sgo1 in the absence of CENP-C. It is interesting that despite the reduction in CENP-T it is still able to recruit similar amounts of KMN proteins and Sgo1 as the full amount of CENP-C. We propose that this could be due to the amount of CENP-T at a normal centromere being in excess of what is required for KMN interactions (Figure D2).

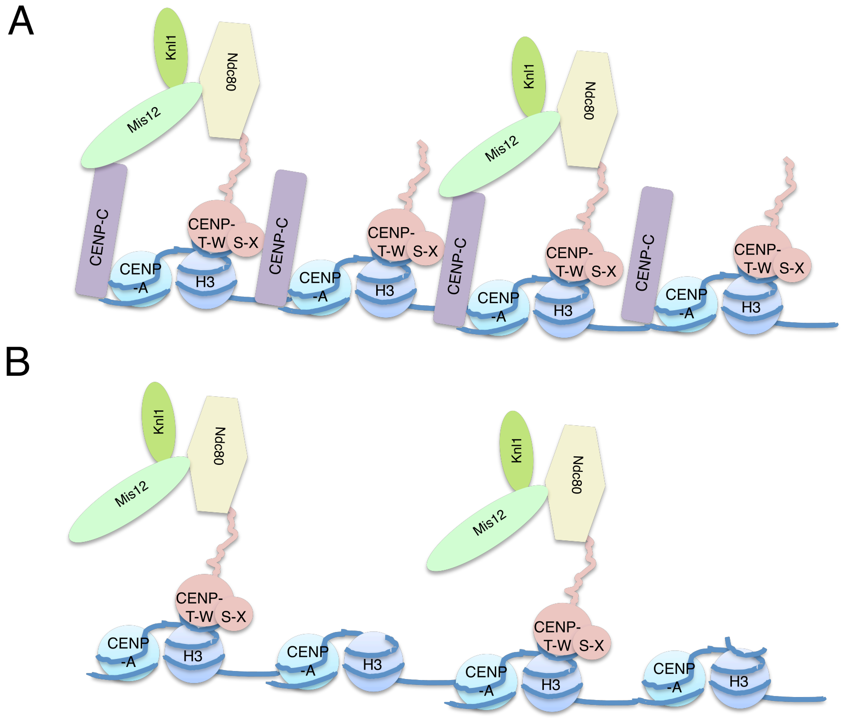


Figure D2: Although CENP-T is reduced with CENP-C depletion it is still able to recruit a substantial amount of the KMN network.

It is known that Aurora B is important in the recruitment of Sgo1. We here showed that the CPC is important in recruitment downstream of both CENP-C and CENP-T. We confirmed that the CPC plays a role in recruitment of the KMN network in Xenopus. This was also recently shown in human cells (Kim and Yu, 2015, Rago et al., 2015). Although in Sgo1 depletion Aurora B does not accumulate at centromeres its normal binding to chromosomes is sufficient for the Mis12 and Ndc80 recruitment at centromeres. Thus it is clear that Aurora B is multifunctional in the Sgo1 recruitment pathway. Using our Bub1 centromere tethering system we would also like to look specifically at whether Aurora B is directly required for Sgo1 recruitment. We will do this by chemically inhibiting Aurora B in the CENP-C depleted CSF chromosomes with the Cen-Bub1 construct added.

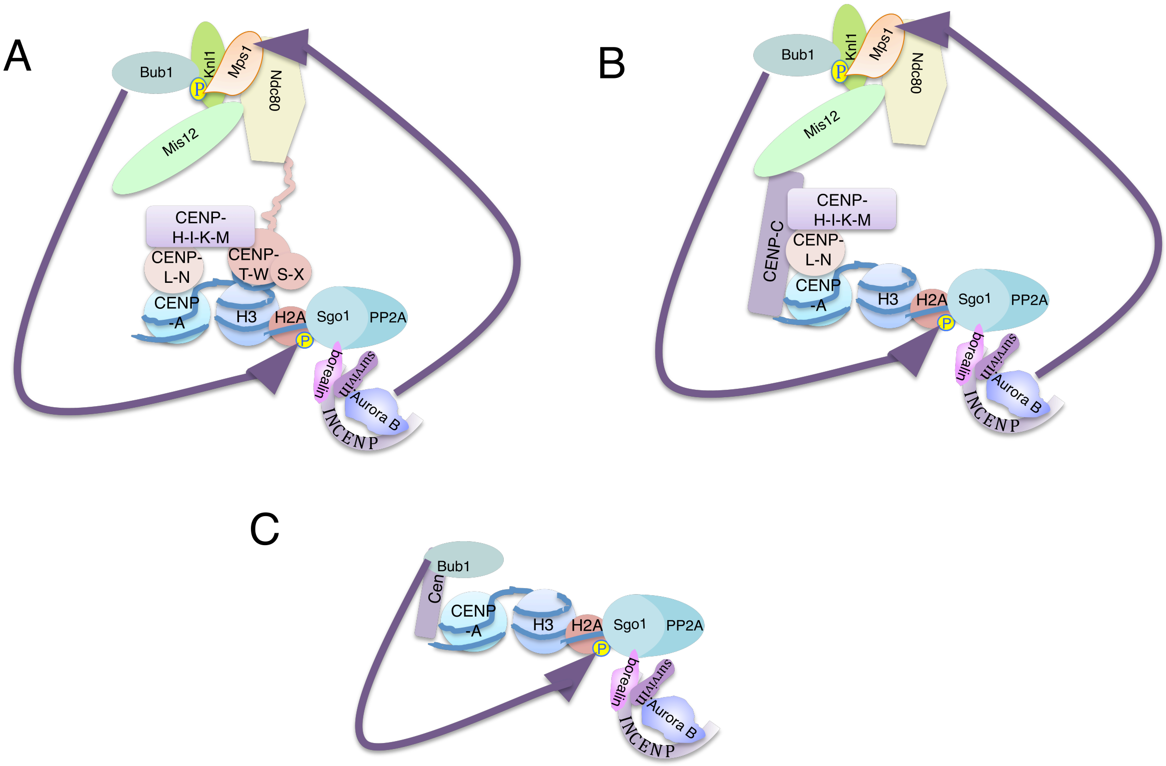


Figure D3: CCAN role in Sgo1 recruitment. (A) Sgo1 recruitment in the absence of CENP-C **(B)** Sgo1 recruitment in the absence of CENP-T **(C)** If Bub1 is artificially tethered to the centromere the requirement for CENP-C or CENP-T is bypassed.

5.3.2 Does Sgo1 have multiple recruitment pathways?

We have shown that the Bub1, as others have seen, is essential for Sgo1 recruitment. We also saw that the only requirement of the kinetochore for Sgo1 recruitment is to recruit Bub1. However there has been other suggested recruitment pathways of Sgo1. CENP-N has been proposed to aid in the recruitment of Sgo1 by direct interaction in yeast (Hinshaw and Harrison, 2013). We showed that CENP-N was not required for Sgo1 recruitment and that CCAN proteins are unlikely to be essential for Sgo1 recruitment other than in forming the base for KMN binding. In CSF chromosomes in which CENP-C has been depleted CENP-N, CENP-K and CENP-T are not present and yet targeting of the Bub1 kinase domain to these centromeres is sufficient to recruit Sgo1. This is supported by data from ectopically formed kinetochores with the N-terminals of CENP-C and CENP-T; they show recruitment of Sgo1 and aurora B with no recruitment of other CCAN proteins (Hori et al., 2013, Gascoigne et al., 2011). However, we have not ruled out that CENP-N contributes to Sgo1 recruitment and it's something that we would like to look into further.

Mps1 depletion or inhibition does not completely remove Sgo1 from the centromere. This may be due to incomplete depletion, as only a small amount of kinase can be sufficient to create a signal great enough for recruitment. However, the other possibility is that there is another recruitment pathway that does not require Mps1 but does require Bub1. A separate pathway that is dependent on CENP-T would be consistent with our results. Although we show that Bub1 phosphorylation is sufficient in the absence of the KMN network there is some evidence to suggest other contributing, although not essential, pathways in Sgo1 recruitment. The reduction we see in Sgo1 in either the CENP-C or CENP-T depletion is not relative to the levels of pH2A in which we see little reduction. As it has recently been shown that Sgo1 directly interacts directly with phosphorylated H2A (Liu et al., 2015). In yeast a phospho-mimic H2A mutant is not sufficient to recruit Sgo1 in the absence of Bub1 (Nerusheva et al., 2014). This suggests that Bub1 may have another target that is required for Sgo1 recruitment. Our results show that if this is the case the other target is unlikely to be one of the KMN network. A search in an online phosphorylation site database (http://www.phosphonetworks.org/) shows CENP-S (also known as APITD1) as a potential target of Bub1. We would like to investigate further whether this is the case and if so whether this phosphorylation contributes to Sgo1 recruitment. However since in CSF chromosomes, in which we did the Bub1 targeting assays, CENP-T is not present it is unlikely CENP-S is present. Therefore, even if CENP-S were a target that helps recruit Sgo1 it would not be essential.

5.3.3 CENP-C in centromeric cohesion

Mitotic cohesion is the result of three consecutive processes:

(1) cohesin deposition before DNA replication;
(2) cohesion establishment during DNA replication;
(3) protection of cohesin from removal in prophase.

Sgo1 recruitment to centromeres in mitosis was compromised in the absence of CENP-C or CENP-T to an extent sufficient for protection. We therefore wondered whether depletion of CENP-C could affect cohesin deposition or cohesion establishment around centromeres. In yeast, enhanced loading of cohesin at centromeres requires the kinetochore Ctf19 complex, the budding yeast CCAN, and is essential for faithful chromosome segregation (Eckert et al., 2007, Fernius and Marston, 2009, Hinshaw et al., 2015). Whether a similar mechanism exists in vertebrate cells is not known. In Xenopus egg extracts, a large fraction of the cohesin loader Scc2-Scc4 is found in association with Dbf4/Drf1-dependent kinase (DDK), thereby ensuring loading of cohesin at sites where DNA replication initiates (Takahashi et al., 2008). However, a small fraction of Scc2-Scc4 is not bound to DDK (20-30%) and could be instead forming a complex with CENP-C. Immunoprecipitation experiments, however, did not support this possibility (T. Takahashi, personal communication). We also compared by immunoblot the amount of cohesin left on mitotic chromosomes obtained from extracts depleted of CENP-C, CENP-T, or mock depleted. A small reduction in cohesin Smc3 could be observed in the CENP-C or CENP-T depleted chromatin compared to control (Figure R36a). Since this reduction was similar in the absence of either CENP-C or CENP-T, it could not explain the different extent of cohesion defects, and was likely the consequence of decreased protection by Sgo1. Immunofluorescence staining of the same chromosomes with a cohesin antibody showed similar results (Figure R36b). Thus, it is unlikely that reduced loading of cohesin at centromeres causes the centromeric cohesion defects that we observe in the absence of CENP-C.

In order to test whether there is a problem in cohesion establishment at centromeres in the absence of CENP-C, we could stain mitotic chromosomes with an antibody against Sororin. This is a cohesin interacting protein that associates specifically with “cohesive” cohesin (Nishiyama et al., 2010). Unfortunately, such a reagent is not available. An alternative possibility, currently under evaluation, is to rescue the cohesion defect observed in the absence of CENP-C by depletion of Wapl. Wapl is responsible for cohesion dissociation in prophase and its action is counteracted by Sororin.

In the course of our experiments, we observed that CENP-A staining was always lower in chromosomes assembled in CENP-C depleted extracts compared to those from mock depleted or CENP-T depleted extracts (Figure 37). We and others previously reported that CENP-C is required for CENP-A deposition upon exit from mitosis (Krizaic et al., 2015, Moree et al., 2011). However, *de novo* deposition of CENP-A in the cell free extract requires that sperm chromatin is first incubated in CSF extracts and then driven into interphase by calcium addition. If chromatin is added to extracts that are already in interphase, then CENP-A deposition does not take place (Bernad et al., 2011). The experiments performed in the current study follow this protocol, and thus the difference in CENP-A levels in chromosomes assembled in mock depleted and CENP-C depleted extract cannot be the result of impaired CENP-A deposition in the absence of CENP-C. Moreover, when we quantitated the intensity of centromeric CENP-A staining in the different depletion conditions the reduction observed in CENP-C depleted extracts was clearly below the 2 fold expected from a simple deposition failure (Figure 37). This result suggests that CENP-C, in addition to promoting incorporation of new CENP-A before DNA replication, contributes to its stabilization within centromeric chromatin. Consistent with our observation, a recent study reports that CENP-C reshapes and stabilizes CENP-A nucleosomes in human cells (Falk et al., 2015). Proper maintenance of CENP-A levels could therefore be important for cohesion. Thus, we would like to assess whether altering the levels of CENP-A at the centromere through depletion of HJURP, the CENP-A loading protein, or even CENP-A itself, we can reproduce the cohesion defects observed in the absence of CENP-C.

Finally, it is conceivable that the increased separation between sister centromeres in CENP-C depleted chromosomes is not the result of defective cohesion. Instead, it could be the consequence of aberrant folding of centromeric chromatin due to a diminished ratio of CENP-A/H3 nucleosomes and/or to the absence of one major component, CENP-C (Ribeiro et al., 2010). The use of superesolution microscopy could help us test this hypothesis.

5.4 Centromeric condensin II

5.4.1 Condensin II recruitment

The shugoshin pathway is not required for condensin II recruitment to the centromere in the Xenopus egg extract system. The role of shugoshin in condensin recruitment has been suggested mainly in yeast. Condensin II has been lost in yeast during evolution, therefore this role of Sgo1 may have been an adaptation when condensin I needed to perform a role previously performed by condensin II. This could explain why we don't see any role of Shugoshin in regulating condensin II. Mps1 phosphorylates CAP-H2 in human cells allowing recruitment to chromosomes. We see no effect of Mps1 depletion on recruitment on condensin II in *Xenopus*. The CPC is clearly required for accumulation at centromeres but not loading on to chromatin, as has been seen in other systems (Ono et al., 2004). It is not clear if the CPC phosphorylates condensin II directly or another component which is required for condensin II recruitment.

We found that CENP-C is required for condensin II recruitment. In human cells it is likely only a percentage of CENP-C binds outer kinetochore proteins (Suzuki et al., 2014) and therefore a large population would be free for other functions. It has previously been seen in *C.elegans* that recruitment of CAP-D3 homologue HCP-6, condensin II specific subunit, but not the SMC2 homologue required CENP-C to be recruited to centromeres. In that system co-depletion of cohesin was able to rescue localisation of condensin II to centromeres in the absence of CENP-C (Moore et al., 2005) we will need to check if this occurs in *Xenopus*. Our results suggested that CENP-C and condensin II could interact directly. If we can identify how CENP-C and condensin II interact we could try to disrupt this interaction by mutation of condensin II and see in other model systems how this affects function.

Our results suggest that other CCAN components are dispensable for condensin II recruitment. It has been seen that CENP-I is important for, condensin II specific subunit, CAP-H2 recruitment to centromere in human cells. When we depleted CENP-K a member of the CENP-H-I-K-M complex we do not see a decrease in CAP-H2 recruitment to the centromere. In the knockdown of CENP-I in human cells the depletion was done over 48 hours during this time the cells can pass through several cell cycle and other errors may occur that contribute to the condensin II loss. Or it could simply be that there is a species difference when it comes to dependency on CENP-I. There are clear differences in the recruitment of condensin II between model systems and decisive pathway of recruitment continues to elude us. It remains an important pathway to decipher and we plan to continue to elucidate this pathway in *Xenopus* then possibly in human, this can help us to gain a better understanding of the differences between systems and the significance of these differences.

5.4.2 Condensin II function at centromeres

In *C.Elegans*, in which condensin II is the dominant condensin, depletion of CENP-C does not prevent condensation. This could suggest that a CENP-C dependent function would be different from its function in condensation (Maddox et al., 2006). In *C.Elegans* CENP-C recruits condensin II to centromeres. If CENP-C is depleted sister centromeres cannot be resolved under conditions that disturb microtubules (Moore et al., 2005). In the Xenopus system depletion of CENP-C and addition of nocodazole does not prevent sister centromere resolution (Wynne and Funabiki, 2015).

If condensin II functions in CENP-A loading at the centromere in *Xenopus* it appears that this function is not conserved in human cells. Our analysis of condensin II function at the centromere has been limited up to this point and is something we wish to expand on. Although functional assays in the *Xenopus* egg extract system are limited we could, for example, look at the structural effect on centromeres in condensin II depleted extracts by super resolution microscopy. We will also expand our studies in to the human cell line system.

5.5 Final remarks

Faithful chromosome segregation is a fundamental process shared between every life form. It has been studied for over 130 years and the mechanisms are still not fully understood. The kinetochore is an extremely complex network of different proteins with a wide range of overlapping functions. There is still much to understand about this macromolecular structure. In particular, the structure and interactions of the components and how these change at different stages of the cell cycle. This insight into the assembly of the kinetochore is necessary for complete understanding of how it performs its functions. It is clear that use of a wide range of model systems is necessary to achieve this goal. The Xenopus egg extract cell-free system is well suited for studying protein recruitment. The results we presented here give us increased insight in to how this complex mass of proteins depend on each other. Furthermore, we have gained increased understanding of how these interactions are important for cohesion and highlighted the need for further work to understand condensin II's presence at the centromere.

6. Conclusions

Conclusions

1. Kinetochore assembly on chromosomes obtained in CSF extracts from unreplicated sperm chromatin is driven exclusively by CENP-C. These kinetochores do not contain CENP-T, CENP-N or CENP-K, which only bind to chromatin during the interphase following exit from the CSF arrest.

2. CENP-T is not required for the binding of CENP-C, CENP-K or CENP-N to centromeres. In turn, CENP-T recruitment only partially depends on CENP-C, but requires CENP-N and CENP-K for stabilisation. A strong interdependency is observed between CENP-N and CENP-K.

3. CENP-C and CENP-T drive two independent pathways of kinetochore assembly that recruit the whole KMN network.

4. CENP-C and CENP-T can independently recruit the cohesin protector Sgo1 to mitotic centromeres.

5. Bub1 targeting to centromeres in the absence of kinetochores is sufficient for Sgo1 recruitment.

6. CENP-C plays a role in centromeric cohesion further to its role in recruitment of Sgo1. The nature of this role remains to be elucidated.

7. CENP-C and the Chromosomal Passenger Complex (CPC), but not Sgo1, are required for accumulation of condensin II at centromeres in mitosis.

Conclusiones

1. La proteína CENP-C dirige por sí sola el ensamblaje del cinetocoro en cromosomas obtenidos de extractos CSF (parados en meiosis II) a partir de cromatina de esperma sin replicar. Estos cinetocoros carecen de CENP-T, CENP-N o CENP-K, que sólo se unen a la cromatina en la interfase posterior.

2. La presencia de CENP-T no es necesaria para la unión de CENP-C, CENP-K o CENP-N a la cromatina centromérica. La unión de CENP-T depende sólo en parte de CENP-C, y en mayor medida, de CENP-K y CENP-N. Estas últimas son muy interdependientes.

3. CENP-C y CENP-T dirigen dos vías independientes para el ensamblaje del cinetocoro, cada una de ellas capaz de atraer a todos los componentes del complejo KMN.

4. La sola presencia de CENP-C o de CENP-T es suficiente para la unión de Sgo1, el protector de cohesina, a la región centromérica.

5. En ausencia de cinetocoros basta con llevar a Bub1 al centrómero para que Sgo1 pueda unirse a la cromatina centromérica.

6. Además de su función en la unión de Sgo1 al centrómero, CENP-C tiene otra función que es importante para la cohesión centromérica, cuya naturaleza aún desconocemos.

7. La acumulación de condensina II en la región centromérica de los cromosomas mitóticos necesita de CENP-C y del complejo de Aurora B (CPC, por *Chromosomal Passenger Complex*), pero no de Sgo1.

7. References

References

Abe, S., Nagasaka, K., Hirayama, Y., Kozuka-Hata, H., Oyama, M., Aoyagi, Y., Obuse, C. and Hirota, T. (2011) 'The initial phase of chromosome condensation requires Cdk1-mediated phosphorylation of the CAP-D3 subunit of condensin II', *Genes Dev,* 25(8), pp. 863-74.

Amano, M., Suzuki, A., Hori, T., Backer, C., Okawa, K., Cheeseman, I. M. and Fukagawa, T. (2009) 'The CENP-S complex is essential for the stable assembly of outer kinetochore structure', *J Cell Biol,* 186(2), pp. 173-82.

Amaro, A. C., Samora, C. P., Holtackers, R., Wang, E., Kingston, I. J., Alonso, M., Lampson, M., McAinsh, A. D. and Meraldi, P. (2010) 'Molecular control of kinetochore-microtubule dynamics and chromosome oscillations', *Nat Cell Biol,* 12(4), pp. 319-29.

Basilico, F., Maffini, S., Weir, J. R., Prumbaum, D., Rojas, A. M., Zimniak, T., De Antoni, A., Jeganathan, S., Voss, B., van Gerwen, S., Krenn, V., Massimiliano, L., Valencia, A., Vetter, I. R., Herzog, F., Raunser, S., Pasqualato, S. and Musacchio, A. (2014) 'The pseudo GTPase CENP-M drives human kinetochore assembly', *Elife,* 3, pp. e02978.

Bernad, R., Sánchez, P., Rivera, T., Rodríguez-Corsino, M., Boyarchuk, E., Vassias, I., Ray-Gallet, D., Arnaoutov, A., Dasso, M., Almouzni, G. and Losada, A. (2011) 'Xenopus HJURP and condensin II are required for CENP-A assembly', *J Cell Biol,* 192(4), pp. 569-82.

Blower, M. D., Sullivan, B. A. and Karpen, G. H. (2002) 'Conserved organization of centromeric chromatin in flies and humans', *Dev Cell,* 2(3), pp. 319-30.

Bodor, D. L., Valente, L. P., Mata, J. F., Black, B. E. and Jansen, L. E. (2013) 'Assembly in G1 phase and long-term stability are unique intrinsic features of CENP-A nucleosomes', *Mol Biol Cell,* 24(7), pp. 923-32.

Boyarchuk, Y., Salic, A., Dasso, M. and Arnaoutov, A. (2007) 'Bub1 is essential for assembly of the functional inner centromere', *J Cell Biol,* 176(7), pp. 919-28.

Carroll, C. W., Milks, K. J. and Straight, A. F. (2010) 'Dual recognition of CENP-A nucleosomes is required for centromere assembly', *J Cell Biol,* 189(7), pp. 1143-55.

Carroll, C. W., Silva, M. C., Godek, K. M., Jansen, L. E. and Straight, A. F. (2009) 'Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N', *Nat Cell Biol,* 11(7), pp. 896-902.

Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M. and Desai, A. (2006) 'The conserved KMN network constitutes the core microtubule-binding site of the kinetochore', *Cell,* 127(5), pp. 983-97.

Cheeseman, I. M., Hori, T., Fukagawa, T. and Desai, A. (2008) 'KNL1 and the CENP-H/I/K complex coordinately direct kinetochore assembly in vertebrates', *Mol Biol Cell,* 19(2), pp. 587-94.

Cheeseman, I. M., Niessen, S., Anderson, S., Hyndman, F., Yates, J. R., Oegema, K. and Desai, A. (2004) 'A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension', *Genes Dev,* 18(18), pp. 2255-68.

Choo, K. H. (2001) 'Domain organization at the centromere and neocentromere', *Dev Cell,* 1(2), pp. 165-77.

Cordova, A., Aaron Straight, and Jan Skotheim. (2015) *Rapid, Inducible degradation of CENP-C suggests novel roles for CENP-C in centromere assembly and maintenance.* Stanford University, https://searchworks.stanford.edu/view/qj087kc1001.

De Wulf, P., McAinsh, A. D. and Sorger, P. K. (2003) 'Hierarchical assembly of the budding yeast kinetochore from multiple subcomplexes', *Genes Dev,* 17(23), pp. 2902-21.

Earnshaw, W. C. and Rothfield, N. (1985) 'Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma', *Chromosoma,* 91(3-4), pp. 313-21.

Eckert, C. A., Gravdahl, D. J. and Megee, P. C. (2007) 'The enhancement of pericentromeric cohesin association by conserved kinetochore components promotes high-fidelity chromosome segregation and is sensitive to microtubule-based tension', *Genes Dev,* 21(3), pp. 278-91.

Emanuele, M. J., Lan, W., Jwa, M., Miller, S. A., Chan, C. S. and Stukenberg, P. T. (2008) 'Aurora B kinase and protein phosphatase 1 have opposing roles in modulating kinetochore assembly', *J Cell Biol,* 181(2), pp. 241-54.

Fachinetti, D., Folco, H. D., Nechemia-Arbely, Y., Valente, L. P., Nguyen, K., Wong, A. J., Zhu, Q., Holland, A. J., Desai, A., Jansen, L. E. and Cleveland, D. W. (2013) 'A two-step mechanism for epigenetic specification of centromere identity and function', *Nat Cell Biol,* 15(9), pp. 1056-66.

Falk, S. J., Guo, L. Y., Sekulic, N., Smoak, E. M., Mani, T., Logsdon, G. A., Gupta, K., Jansen, L. E., Van Duyne, G. D., Vinogradov, S. A., Lampson, M. A. and Black, B. E. (2015) 'Chromosomes. CENP-C reshapes and stabilizes CENP-A nucleosomes at the centromere', *Science,* 348(6235), pp. 699-703.

Fernius, J. and Marston, A. L. (2009) 'Establishment of cohesion at the pericentromere by the Ctf19 kinetochore subcomplex and the replication fork-associated factor, Csm3', *PLoS Genet,* 5(9), pp. e1000629.

Foltz, D. R., Jansen, L. E., Black, B. E., Bailey, A. O., Yates, J. R. and Cleveland, D. W. (2006) 'The human CENP-A centromeric nucleosome-associated complex', *Nat Cell Biol,* 8(5), pp. 458-69.

Fujiwara, T., Tanaka, K., Kuroiwa, T. and Hirano, T. (2013) 'Spatiotemporal dynamics of condensins I and II: evolutionary insights from the primitive red alga Cyanidioschyzon merolae', *Mol Biol Cell,* 24(16), pp. 2515-27.

Fukagawa, T., Mikami, Y., Nishihashi, A., Regnier, V., Haraguchi, T., Hiraoka, Y., Sugata, N., Todokoro, K., Brown, W. and Ikemura, T. (2001) 'CENP-H, a constitutive centromere component, is required for centromere targeting of CENP-C in vertebrate cells', *EMBO J,* 20(16), pp. 4603-17.

Gascoigne, K. E., Takeuchi, K., Suzuki, A., Hori, T., Fukagawa, T. and Cheeseman, I. M. (2011) 'Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes', *Cell,* 145(3), pp. 410-22.

Gerlich, D., Hirota, T., Koch, B., Peters, J. M. and Ellenberg, J. (2006) 'Condensin I stabilizes chromosomes mechanically through a dynamic interaction in live cells', *Curr Biol,* 16(4), pp. 333-44.

Hagstrom, K. A., Holmes, V. F., Cozzarelli, N. R. and Meyer, B. J. (2002) 'C. elegans condensin promotes mitotic chromosome architecture, centromere organization, and sister chromatid segregation during mitosis and meiosis', *Genes Dev,* 16(6), pp. 729-42.

Hellwig, D., Emmerth, S., Ulbricht, T., Döring, V., Hoischen, C., Martin, R., Samora, C. P., McAinsh, A. D., Carroll, C. W., Straight, A. F., Meraldi, P. and Diekmann, S. (2011) 'Dynamics of CENP-N kinetochore binding during the cell cycle', *J Cell Sci,* 124(Pt 22), pp. 3871-83.

Hemmerich, P., Weidtkamp-Peters, S., Hoischen, C., Schmiedeberg, L., Erliandri, I. and Diekmann, S. (2008) 'Dynamics of inner kinetochore assembly and maintenance in living cells', *J Cell Biol,* 180(6), pp. 1101-14.

Hinshaw, S. M. and Harrison, S. C. (2013) 'An Iml3-Chl4 heterodimer links the core centromere to factors required for accurate chromosome segregation', *Cell Rep,* 5(1), pp. 29-36.

Hinshaw, S. M., Makrantoni, V., Kerr, A., Marston, A. L. and Harrison, S. C. (2015) 'Structural evidence for Scc4-dependent localization of cohesin loading', *Elife,* 4, pp. e06057.

Hirota, T., Gerlich, D., Koch, B., Ellenberg, J. and Peters, J. M. (2004) 'Distinct functions of condensin I and II in mitotic chromosome assembly', *J Cell Sci,* 117(Pt 26), pp. 6435-45.

Holland, A. J., Fachinetti, D., Han, J. S. and Cleveland, D. W. (2012) 'Inducible, reversible system for the rapid and complete degradation of proteins in mammalian cells', *Proc Natl Acad Sci U S A,* 109(49), pp. E3350-7.

Hori, T., Amano, M., Suzuki, A., Backer, C. B., Welburn, J. P., Dong, Y., McEwen, B. F., Shang, W. H., Suzuki, E., Okawa, K., Cheeseman, I. M. and Fukagawa, T. (2008) 'CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore', *Cell,* 135(6), pp. 1039-52.

Hori, T., Shang, W. H., Takeuchi, K. and Fukagawa, T. (2013) 'The CCAN recruits CENP-A to the centromere and forms the structural core for kinetochore assembly', *J Cell Biol,* 200(1), pp. 45-60.

Izuta, H., Ikeno, M., Suzuki, N., Tomonaga, T., Nozaki, N., Obuse, C., Kisu, Y., Goshima, N., Nomura, F., Nomura, N. and Yoda, K. (2006) 'Comprehensive analysis of the ICEN (Interphase Centromere Complex) components enriched in the CENP-A chromatin of human cells', *Genes Cells,* 11(6), pp. 673-84.

Kagami, Y., Nihira, K., Wada, S., Ono, M., Honda, M. and Yoshida, K. (2014) 'Mps1 phosphorylation of condensin II controls chromosome condensation at the onset of mitosis', *J Cell Biol,* 205(6), pp. 781-90.

Kawashima, S. A., Yamagishi, Y., Honda, T., Ishiguro, K. and Watanabe, Y. (2010) 'Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin', *Science,* 327(5962), pp. 172-7.

Kim, S. and Yu, H. (2015) 'Multiple assembly mechanisms anchor the KMN spindle checkpoint platform at human mitotic kinetochores', *J Cell Biol,* 208(2), pp. 181-96.

Kitajima, T. S., Hauf, S., Ohsugi, M., Yamamoto, T. and Watanabe, Y. (2005) 'Human Bub1 defines the persistent cohesion site along the mitotic chromosome by affecting Shugoshin localization', *Curr Biol,* 15(4), pp. 353-9.

Kitajima, T. S., Sakuno, T., Ishiguro, K., Iemura, S., Natsume, T., Kawashima, S. A. and Watanabe, Y. (2006) 'Shugoshin collaborates with protein phosphatase 2A to protect cohesin', *Nature,* 441(7089), pp. 46-52.

Klare, K., Weir, J. R., Basilico, F., Zimniak, T., Massimiliano, L., Ludwigs, N., Herzog, F. and Musacchio, A. (2015) 'CENP-C is a blueprint for constitutive centromere-associated network assembly within human kinetochores', *J Cell Biol,* 210(1), pp. 11-22.

Krenn, V., Wehenkel, A., Li, X., Santaguida, S. and Musacchio, A. (2012) 'Structural analysis reveals features of the spindle checkpoint kinase Bub1-kinetochore subunit Knl1 interaction', *J Cell Biol,* 196(4), pp. 451-67.

Krizaic, I., Williams, S. J., Sánchez, P., Rodríguez-Corsino, M., Stukenberg, P. T. and Losada, A. (2015) 'The distinct functions of CENP-C and CENP-T/W in centromere propagation and function in Xenopus egg extracts', *Nucleus,* 6(2), pp. 133-43.

Kwon, M. S., Hori, T., Okada, M. and Fukagawa, T. (2007) 'CENP-C is involved in chromosome segregation, mitotic checkpoint function, and kinetochore assembly', *Mol Biol Cell,* 18(6), pp. 2155-68.

Lam, W. W., Peterson, E. A., Yeung, M. and Lavoie, B. D. (2006) 'Condensin is required for chromosome arm cohesion during mitosis', *Genes Dev,* 20(21), pp. 2973-84.

Liu, H., Qu, Q., Warrington, R., Rice, A., Cheng, N. and Yu, H. (2015) 'Mitotic Transcription Installs Sgo1 at Centromeres to Coordinate Chromosome Segregation', *Mol Cell,* 59(3), pp. 426-36.

Liu, S. T., Rattner, J. B., Jablonski, S. A. and Yen, T. J. (2006) 'Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells', *J Cell Biol,* 175(1), pp. 41-53.

Lohka, M. J. and Maller, J. L. (1985) 'Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts', *J Cell Biol,* 101(2), pp. 518-23.

Lohka, M. J. and Masui, Y. (1983) 'Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components', *Science,* 220(4598), pp. 719-21.

Losada, A., Hirano, M. and Hirano, T. (1998) 'Identification of Xenopus SMC protein complexes required for sister chromatid cohesion', *Genes Dev,* 12(13), pp. 1986-97.

Maddox, P. S., Portier, N., Desai, A. and Oegema, K. (2006) 'Molecular analysis of mitotic chromosome condensation using a quantitative time-resolved fluorescence microscopy assay', *Proc Natl Acad Sci U S A,* 103(41), pp. 15097-102.

Malvezzi, F., Litos, G., Schleiffer, A., Heuck, A., Mechtler, K., Clausen, T. and Westermann, S. (2013) 'A structural basis for kinetochore recruitment of the Ndc80 complex via two distinct centromere receptors', *EMBO J,* 32(3), pp. 409-23.

Maresca, T. J. and Heald, R. (2006) 'The long and the short of it: linker histone H1 is required for metaphase chromosome compaction', *Cell Cycle,* 5(6), pp. 589-91.

Marston, A. L., Tham, W. H., Shah, H. and Amon, A. (2004) 'A genome-wide screen identifies genes required for centromeric cohesion', *Science,* 303(5662), pp. 1367-70.

McCleland, M. L., Gardner, R. D., Kallio, M. J., Daum, J. R., Gorbsky, G. J., Burke, D. J. and Stukenberg, P. T. (2003) 'The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity', *Genes Dev,* 17(1), pp. 101-14.

McClelland, S. E., Borusu, S., Amaro, A. C., Winter, J. R., Belwal, M., McAinsh, A. D. and Meraldi, P. (2007) 'The CENP-A NAC/CAD kinetochore complex controls chromosome congression and spindle bipolarity', *EMBO J,* 26(24), pp. 5033-47.

McGuinness, B. E., Hirota, T., Kudo, N. R., Peters, J. M. and Nasmyth, K. (2005) 'Shugoshin prevents dissociation of cohesin from centromeres during mitosis in vertebrate cells', *PLoS Biol,* 3(3), pp. e86.

McKinley, K. L., Sekulic, N., Guo, L. Y., Tsinman, T., Black, B. E. and Cheeseman, I. M. (2015) 'The CENP-L-N Complex Forms a Critical Node in an Integrated Meshwork of Interactions at the Centromere-Kinetochore Interface', *Mol Cell,* 60(6), pp. 886-98.

Milks, K. J., Moree, B. and Straight, A. F. (2009) 'Dissection of CENP-C-directed centromere and kinetochore assembly', *Mol Biol Cell,* 20(19), pp. 4246-55.

Moore, L. L., Stanvitch, G., Roth, M. B. and Rosen, D. (2005) 'HCP-4/CENP-C promotes the prophase timing of centromere resolution by enabling the centromere association of HCP-6 in Caenorhabditis elegans', *Mol Cell Biol,* 25(7), pp. 2583-92.

Moree, B., Meyer, C. B., Fuller, C. J. and Straight, A. F. (2011) 'CENP-C recruits M18BP1 to centromeres to promote CENP-A chromatin assembly', *J Cell Biol,* 194(6), pp. 855-71.

Nakazawa, N., Nakamura, T., Kokubu, A., Ebe, M., Nagao, K. and Yanagida, M. (2008) 'Dissection of the essential steps for condensin accumulation at kinetochores and rDNAs during fission yeast mitosis', *J Cell Biol,* 180(6), pp. 1115-31.

Nerusheva, O. O., Galander, S., Fernius, J., Kelly, D. and Marston, A. L. (2014) 'Tension-dependent removal of pericentromeric shugoshin is an indicator of sister chromosome biorientation', *Genes Dev,* 28(12), pp. 1291-309.

Nishino, T., Rago, F., Hori, T., Tomii, K., Cheeseman, I. M. and Fukagawa, T. (2013) 'CENP-T provides a structural platform for outer kinetochore assembly', *EMBO J,* 32(3), pp. 424-36.

Nishiyama, T., Ladurner, R., Schmitz, J., Kreidl, E., Schleiffer, A., Bhaskara, V., Bando, M., Shirahige, K., Hyman, A. A., Mechtler, K. and Peters, J. M. (2010) 'Sororin mediates sister chromatid cohesion by antagonizing Wapl', *Cell,* 143(5), pp. 737-49.

Obuse, C., Yang, H., Nozaki, N., Goto, S., Okazaki, T. and Yoda, K. (2004) 'Proteomics analysis of the centromere complex from HeLa interphase cells: UV-damaged DNA binding protein 1 (DDB-1) is a component of the CEN-complex, while BMI-1 is transiently co-localized with the centromeric region in interphase', *Genes Cells,* 9(2), pp. 105-20.

Okada, M., Cheeseman, I. M., Hori, T., Okawa, K., McLeod, I. X., Yates, J. R., Desai, A. and Fukagawa, T. (2006) 'The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres', *Nat Cell Biol,* 8(5), pp. 446-57.

Oliveira, R. A., Coelho, P. A. and Sunkel, C. E. (2005) 'The condensin I subunit Barren/CAP-H is essential for the structural integrity of centromeric heterochromatin during mitosis', *Mol Cell Biol,* 25(20), pp. 8971-84.

Ono, T., Fang, Y., Spector, D. L. and Hirano, T. (2004) 'Spatial and temporal regulation of Condensins I and II in mitotic chromosome assembly in human cells', *Mol Biol Cell,* 15(7), pp. 3296-308.

Ono, T., Losada, A., Hirano, M., Myers, M. P., Neuwald, A. F. and Hirano, T. (2003) 'Differential contributions of condensin I and condensin II to mitotic chromosome architecture in vertebrate cells', *Cell,* 115(1), pp. 109-21.

Ono, T., Yamashita, D. and Hirano, T. (2013) 'Condensin II initiates sister chromatid resolution during S phase', *J Cell Biol,* 200(4), pp. 429-41.

Peplowska, K., Wallek, A. U. and Storchova, Z. (2014) 'Sgo1 regulates both condensin and Ipl1/Aurora B to promote chromosome biorientation', *PLoS Genet,* 10(6), pp. e1004411.

Perpelescu, M. and Fukagawa, T. (2011) 'The ABCs of CENPs', *Chromosoma,* 120(5), pp. 425-46.

Petrovic, A., Pasqualato, S., Dube, P., Krenn, V., Santaguida, S., Cittaro, D., Monzani, S., Massimiliano, L., Keller, J., Tarricone, A., Maiolica, A., Stark, H. and Musacchio, A. (2010) 'The MIS12 complex is a protein interaction hub for outer kinetochore assembly', *J Cell Biol,* 190(5), pp. 835-52.

Pfau, S. J. and Amon, A. (2012) 'Chromosomal instability and aneuploidy in cancer: from yeast to man', *EMBO Rep,* 13(6), pp. 515-27.

Philpott, A. and Leno, G. H. (1992) 'Nucleoplasmin remodels sperm chromatin in Xenopus egg extracts', *Cell,* 69(5), pp. 759-67.

Prendergast, L., van Vuuren, C., Kaczmarczyk, A., Doering, V., Hellwig, D., Quinn, N., Hoischen, C., Diekmann, S. and Sullivan, K. F. (2011) 'Premitotic assembly of human CENPs -T and -W switches centromeric chromatin to a mitotic state', *PLoS Biol,* 9(6), pp. e1001082.

Przewloka, M. R., Venkei, Z., Bolanos-Garcia, V. M., Debski, J., Dadlez, M. and Glover, D. M. (2011) 'CENP-C is a structural platform for kinetochore assembly', *Curr Biol,* 21(5), pp. 399-405.

Rago, F., Gascoigne, K. E. and Cheeseman, I. M. (2015) 'Distinct organization and regulation of the outer kinetochore KMN network downstream of CENP-C and CENP-T', *Curr Biol,* 25(5), pp. 671-7.

Ribeiro, S. A., Vagnarelli, P., Dong, Y., Hori, T., McEwen, B. F., Fukagawa, T., Flors, C. and Earnshaw, W. C. (2010) 'A super-resolution map of the vertebrate kinetochore', *Proc Natl Acad Sci U S A,* 107(23), pp. 10484-9.

Riedel, C. G., Katis, V. L., Katou, Y., Mori, S., Itoh, T., Helmhart, W., Gálová, M., Petronczki, M., Gregan, J., Cetin, B., Mudrak, I., Ogris, E., Mechtler, K., Pelletier, L., Buchholz, F., Shirahige, K. and Nasmyth, K. (2006) 'Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I', *Nature,* 441(7089), pp. 53-61.

Rivera, T. and Losada, A. (2009) 'Shugoshin regulates cohesion by driving relocalization of PP2A in Xenopus extracts', *Chromosoma,* 118(2), pp. 223-33.

Sakamoto, T., Inui, Y. T., Uraguchi, S., Yoshizumi, T., Matsunaga, S., Mastui, M., Umeda, M., Fukui, K. and Fujiwara, T. (2011) 'Condensin II alleviates DNA damage and is essential for tolerance of boron overload stress in Arabidopsis', *Plant Cell,* 23(9), pp. 3533-46.

Salic, A., Waters, J. C. and Mitchison, T. J. (2004) 'Vertebrate shugoshin links sister centromere cohesion and kinetochore microtubule stability in mitosis', *Cell,* 118(5), pp. 567-78.

Samejima, I., Spanos, C., Alves, F. e. L., Hori, T., Perpelescu, M., Zou, J., Rappsilber, J., Fukagawa, T. and Earnshaw, W. C. (2015) 'Whole-proteome genetic analysis of dependencies in assembly of a vertebrate kinetochore', *J Cell Biol,* 211(6), pp. 1141-56.

Samoshkin, A., Arnaoutov, A., Jansen, L. E., Ouspenski, I., Dye, L., Karpova, T., McNally, J., Dasso, M., Cleveland, D. W. and Strunnikov, A. (2009) 'Human condensin function is essential for centromeric chromatin assembly and proper sister kinetochore orientation', *PLoS One,* 4(8), pp. e6831.

Saurin, A. T., van der Waal, M. S., Medema, R. H., Lens, S. M. and Kops, G. J. (2011) 'Aurora B potentiates Mps1 activation to ensure rapid checkpoint establishment at the onset of mitosis', *Nat Commun,* 2, pp. 316.

Schleiffer, A., Maier, M., Litos, G., Lampert, F., Hornung, P., Mechtler, K. and Westermann, S. (2012) 'CENP-T proteins are conserved centromere receptors of the Ndc80 complex', *Nat Cell Biol,* 14(6), pp. 604-13.

Suzuki, A., Badger, B. L., Wan, X., DeLuca, J. G. and Salmon, E. D. (2014) 'The architecture of CCAN proteins creates a structural integrity to resist spindle forces and achieve proper Intrakinetochore stretch', *Dev Cell,* 30(6), pp. 717-30.

Takahashi, K., Chen, E. S. and Yanagida, M. (2000) 'Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast', *Science,* 288(5474), pp. 2215-9.

Takahashi, T. S., Basu, A., Bermudez, V., Hurwitz, J. and Walter, J. C. (2008) 'Cdc7-Drf1 kinase links chromosome cohesion to the initiation of DNA replication in Xenopus egg extracts', *Genes Dev,* 22(14), pp. 1894-905.

Takemoto, A., Maeshima, K., Ikehara, T., Yamaguchi, K., Murayama, A., Imamura, S., Imamoto, N., Yokoyama, S., Hirano, T., Watanabe, Y., Hanaoka, F., Yanagisawa, J. and Kimura, K. (2009) 'The chromosomal association of condensin II is regulated by a noncatalytic function of PP2A', *Nat Struct Mol Biol,* 16(12), pp. 1302-8.

Tanaka, K., Chang, H. L., Kagami, A. and Watanabe, Y. (2009) 'CENP-C functions as a scaffold for effectors with essential kinetochore functions in mitosis and meiosis', *Dev Cell,* 17(3), pp. 334-43.

Tang, Z., Shu, H., Qi, W., Mahmood, N. A., Mumby, M. C. and Yu, H. (2006) 'PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation', *Dev Cell,* 10(5), pp. 575-85.

Tang, Z., Sun, Y., Harley, S. E., Zou, H. and Yu, H. (2004) 'Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis', *Proc Natl Acad Sci U S A,* 101(52), pp. 18012-7.

Uhlmann, F., Lottspeich, F. and Nasmyth, K. (1999) 'Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1', *Nature,* 400(6739), pp. 37-42.

Verzijlbergen, K. F., Nerusheva, O. O., Kelly, D., Kerr, A., Clift, D., de Lima Alves, F., Rappsilber, J. and Marston, A. L. (2014) 'Shugoshin biases chromosomes for biorientation through condensin recruitment to the pericentromere', *Elife,* 3, pp. e01374.

Vigneron, S., Prieto, S., Bernis, C., Labbé, J. C., Castro, A. and Lorca, T. (2004) 'Kinetochore localization of spindle checkpoint proteins: who controls whom?', *Mol Biol Cell,* 15(10), pp. 4584-96.

Vleugel, M., Tromer, E., Omerzu, M., Groenewold, V., Nijenhuis, W., Snel, B. and Kops, G. J. (2013) 'Arrayed BUB recruitment modules in the kinetochore scaffold KNL1 promote accurate chromosome segregation', *J Cell Biol,* 203(6), pp. 943-55.

Westhorpe, F. G. and Straight, A. F. (2015) 'The centromere: epigenetic control of chromosome segregation during mitosis', *Cold Spring Harb Perspect Biol,* 7(1), pp. a015818.

Wood, A. J., Severson, A. F. and Meyer, B. J. (2010) 'Condensin and cohesin complexity: the expanding repertoire of functions', *Nat Rev Genet,* 11(6), pp. 391-404.

Wood, L., Booth, D. G., Vargiu, G., Ohta, S., deLima Alves, F., Samejima, K., Fukagawa, T., Rappsilber, J. and Earnshaw, W. C. (2016) 'Auxin/AID versus conventional knockouts: distinguishing the roles of CENP-T/W in mitotic kinetochore assembly and stability', *Open Biol,* 6(1), pp. 150230.

Wynne, D. J. and Funabiki, H. (2015) 'Kinetochore function is controlled by a phospho-dependent coexpansion of inner and outer components', *J Cell Biol,* 210(6), pp. 899-916.

Yamagishi, Y., Sakuno, T., Shimura, M. and Watanabe, Y. (2008) 'Heterochromatin links to centromeric protection by recruiting shugoshin', *Nature,* 455(7210), pp. 251-5.

Yamagishi, Y., Yang, C. H., Tanno, Y. and Watanabe, Y. (2012) 'MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components', *Nat Cell Biol,* 14(7), pp. 746-52.

Zeitlin, S. G., Patel, S., Kavli, B. and Slupphaug, G. (2005) 'Xenopus CENP-A assembly into chromatin requires base excision repair proteins', *DNA Repair (Amst),* 4(7), pp. 760-72.